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EXPERIMENTAL NEUROTROPHIC THERAPIES OF PARKINSON'S DISEASE: EFFECTS ON NIGROSTRIATAL DOPAMINE SYSTEM

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“Kites rise highest against the wind, not with it.”
— Sir Winston Churchill

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ABSTRACT

Parkinson's disease (PD) is a chronic neurodegenerative disorder characterized by progressive loss of nigrostriatal dopamine neurons and propagating Lewy body pathology. Dopamine depletion in the striatum gives rise to the cardinal motor symptoms of PD. Current PD medications are based on replenishing striatal dopamine and provide symptomatic relief to the motor deficits. However, troublesome adverse effects and diminished efficacy complicate their long-term use. There is a great unmet medical need for a therapy that could slow or halt the progression of the disease. Neurotrophic factors (NTFs) are secreted proteins that promote neuronal growth, differentiation and survival. They are able to prevent the progression of neurodegeneration and restore aberrant neuronal function in a variety of preclinical models of PD. Nonetheless, outcomes from clinical trials have been disappointing. The purpose of this work was to characterize the effects of cerebral dopamine neurotrophic factor (CDNF), mesencephalic astrocyte-derived neurotrophic factor (MANF) and novel small molecule receptor tyrosine kinase RET agonists (BT13 and BT44) on nigrostriatal dopamine system and support their preclinical development as potential neurotrophic therapies of PD.

To further clarify the functional effects of glial cell line-derived neurotrophic factor (GDNF), CDNF and MANF in the normal rat brain, microdialysis measurements were performed after a bolus injection of NTFs into the striatum. We saw augmented stimulus-evoked dopamine release and elevated dopamine turnover in the striatum of MANF-injected rats. GDNF injection increased *in vivo* tyrosine hydroxylase (TH) and catechol-*O*-methyltransferase activity and decreased monoamine oxidase A activity. These data are relevant when considering exogenously administered NTFs as a potential therapeutic approach for PD, since they have to be compatible with the existing dopaminergic medications of the patients.

We also investigated the distribution properties of ^{125}I-labeled and unlabeled CDNF after a nigral injection to intact rats. CDNF readily diffused into the brain areas surrounding the injection site and colocalized with TH-immunoreactive neurons in the substantia nigra. We did not detect active transportation of CDNF to distal brain areas. This characterization provides valuable insights into the selection of optimal delivery site and protocol for CDNF therapy.

Our *in vitro* assays showed that RET agonists BT13 and BT44 were able to induce RET phosphorylation and activate downstream pro-survival signaling cascades Akt and ERK. They also supported the survival of cultured midbrain dopamine neurons from wild-type, but not from RET knockout, mice. The functional effects of BT13 and BT44 were evaluated in a unilateral 6-hydroxydopamine rat model of PD, where both compounds alleviated amphetamine-induced turning behavior. BT44 also showed potential to restore striatal TH-immunoreactive fibers. As blood-brain barrier penetrating compounds, BT13 and BT44 serve as promising leads that can be further developed into a disease-modifying therapy for PD.

TIIVISTELMÄ

Parkinsonin tauti on etenevä hermorappeumasairaus, jolle on ominaista aivojen mustatumakkeen dopamiinihermosolujen tuhoutuminen ja Lewyn kappaleiden esiintyminen aivoissa. Dopamiinihermosolujen tuhoutuminen johtaa aivojuovion dopamiinivajeeseen, joka saa aikaan Parkinsonin taudille ominaiset liikehäiriöt. Taudin nykyinen lääkitys perustuu dopamiinivajeen korjaamiseen ja on luonteeltaan oireita lievittävää. Pitkäaikaiskäytössä lääkkeet menettävät tehoaan ja johtavat hankaliin haittavaikutuksiin. Taudin kulkua hidastavalle hoidolle onkin suuri tarve. Hermokasvutekijät ovat hermosolujen kasvua, erilaistumista ja selviytymistä edistäviä proteiineja. Niiden on osoitettu estävän dopamiinihermosolujen tuhoutumista ja korjaavan niiden häiriintynyttä toimintaa useissa kokeellisissa Parkinson-malleissa. Kliinisissä kokeissa testattujen hermokasvutekijähoitojen teho on kuitenkin jäänyt puutteelliseksi. Tämän väitöstyön tarkoituksena oli selvittää CDNF:n (dopamiinisolujen hermokasvutekijä) ja MANF:n (keskiaivojen astrosyyttiperäinen hermokasvutekijä) sekä uusien, RET-tyrosiinikinaasia aktivoivien pienmolekyylien BT13:n ja BT44:n vaikutuksia aivojen dopamiinijärjestelmään ja samalla tukea niiden prekliinistä kehitystä mahdolliseksi Parkinsonin taudin kulkuun vaikuttavaksi hoidoksi.

Ensimmäisessä osatyössä selvitimme aivojuovioon annostellun GDNF:n (gliasolulinjaperäinen hermokasvutekijä), CDNF:n ja MANF:n aiheuttamia toiminnallisia muutoksia terveen rotan aivoissa käyttämällä hyväksi mikrodialyysimenetelmää. Havaitsimme MANF:n lisäävän stimuloitua dopamiinin vapautumista ja dopamiinin aineenvaihduntaa aivojuoviossa. GDNF injektio puolestaan lisäsi dopamiinia syntetisoivan tyrosiinihydroksylaasientsyymin aktiivisuutta. Lisäksi se vaikutti dopamiinia metaboloivien entsyymien toimintaan lisäämällä katekoli-*O*-metyylitransferaasin ja vähentämällä monoamiinioksidaasin aktiivisuutta. Nämä löydökset auttavat sovittamaan mahdollisia hermokasvutekijähoitoja yhteen Parkinson-potilaiden käyttämien dopaminergisten lääkkeiden kanssa.

Toisessa osatyössä tutkimme CDNF:n leviämisominaisuuksia terveen rotan aivoissa mustatumakkeeseen kohdistuneen annostelun jälkeen. CDNF levisi laajalle injektiokohtaa ympäröivään aivokudokseen ja oli havaittavissa mustatumakkeen dopamiinihermosoluissa. Merkkejä CDNF:n aktiivisesta kuljetuksesta kaukaisemmille aivoalueille ei havaittu. Tämä tutkimus tarjoaa arvokasta lisätietoa CDNF:n optimaalisen annostelutavan määrittämiseksi.

Kolmannessa ja neljännessä osatyössä osoitimme BT13:n ja BT44:n aktivoivan soluja suojaavat Akt- ja ERK-signalointireitit sekä edistävän viljeltyjen dopamiinihermosolujen selviytymistä. Kun BT13:n ja BT44:n vaikutuksia tutkittiin Parkinsonin taudin eläinmallissa rotilla, molempien yhdisteiden havaittiin lievittävän kokeessa mitattua liikehäiriötä. BT44 osoitti myös viitteitä dopaminergisia hermosäikeitä korjaavasta vaikutuksesta. Veri-aivoesteen läpäisevät BT13 ja BT44 ovatkin lupaavia johtolankamolekyylejä, joita edelleen optimoimalla voisi olla mahdollista kehittää uusi Parkinsonin taudin etenemistä hidastava hoito.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Renko J-M, Bäck S, Voutilainen MH, Piepponen TP, Reenilä I, Saarma M, Tuominen RK (2018) Mesencephalic Astrocyte-Derived Neurotrophic Factor (MANF) Elevates Stimulus-Evoked Release of Dopamine in Freely-Moving Rats. Mol. Neurobiol. 55(8):6755-6768

II Albert K, Renko J-M, Mätlik K, Airavaara M, Voutilainen MH (2019) Cerebral Dopamine Neurotrophic Factor Diffuses Around the Brainstem and Does Not Undergo Anterograde Transport After Injection to the Substantia Nigra. Front. Neurosci. 13:590

III Mahato AK*, Renko J-M*, Kopra J, Visnapuu T, Korhonen I, Pulkkinen N, Bespalov M, Ronken E, Piepponen TP, Voutilainen MH, Tuominen RK, Karelson M, Sidorova YA, Saarma M (2019) GDNF receptor agonist supports dopamine neurons in vitro and protects their function in animal model of Parkinson's disease. bioRxiv 540021, *Preprint manuscript*

IV Renko J-M*, Mahato AK*, Visnapuu T, Karelson M, Voutilainen MH, Saarma M, Tuominen RK, Sidorova YA (2020) Neuroprotective potential of a small molecule RET agonist in cultured dopamine neurons and hemiparkinsonian rats. *Manuscript under review*

* Equal contribution

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ABBREVIATIONS

3-MT	3-Methoxytyramine
6-OHDA	6-Hydroxydopamine
AADC	Aromatic amino acid decarboxylase
AAV	Adeno-associated virus
AD	Alzheimer's disease
ANOVA	Analysis of variance
ARTN	Artemin
ATP	Adenosine triphosphate
α-syn	α-Synuclein
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
CDNF	Cerebral dopamine neurotrophic factor
CNS	Central nervous system
COMT	Catechol-*O*-methyltransferase
CSF	Cerebrospinal fluid
DAT	Dopamine transporter
DOPAC	3,4-Dihydroxyphenylacetic acid
ENS	Enteric nervous system
ER	Endoplasmic reticulum
^{18}F-DOPA	6-^{18}F-fluoro-L-dopa
FGF	Fibroblast growth factor
GABA	γ-Aminobutyric acid
GDNF	Glial cell line-derived neurotrophic factor
GFL	GDNF family ligand
GFP	Green fluorescent protein
GFRα	GDNF family receptor α
GPe	Globus pallidus external segment
GPi	Globus pallidus internal segment
GPI	Phosphatidylinositol
GRP78	Glucose-regulated protein 78 kDa
HPLC	High-performance liquid chromatography
HVA	Homovanillic acid
i.p.	Intraperitoneally
ir	Immunoreactive
L-DOPA	L-3,4-Dihydroxyphenylalanine
LPS	Lipopolysaccharide
LTD	Long-term depression
LTP	Long-term potentiation
MANF	Mesencephalic astrocyte-derived neurotrophic factor

MAO	Monoamine oxidase
MAPK	Mitogen activated protein kinase
MCI	Mild cognitive impairment
MPP+	1-Methyl-4-phenylpyridinium
MPTP	1-Methyl-4-phenyl-1,2,3,6- tetrahydropyridine
MRI	Magnetic resonance imaging
MSN	Medium spiny neuron
NAcc	Nucleus accumbens
nAChR	Nicotinic acetylcholine receptor
NCAM	Neural cell adhesion molecule
NGF	Nerve growth factor
NHP	Non-human primate
NRTN	Neurturin
NTF	Neurotrophic factor
PBS	Phosphate-buffered saline
PD	Parkinson's disease
PET	Positron emission tomography
PG	Propylene glycol
PNS	Peripheral nervous system
PSPN	Persephin
PV	Parvalbumin
REGWF	Ryan-Einot-Gabriel-Welsch F
RET	Rearranged during transfection
RMS	Rostral migratory stream
ROS	Reactive oxygen species
s.c.	Subcutaneous
SEM	Standard error of the mean
SGZ	Subgranular zone
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
SPECT	Single-photon emission computed tomography
STN	Subthalamic nucleus
SVZ	Subventricular zone
TGF-β	Transforming growth factor β
TH	Tyrosine hydroxylase
UPDRS	Unified Parkinson's disease rating scale
UPR	Unfolded protein response
VMAT2	Vesicular monoamine transporter 2
VTA	Ventral tegmental area

1 INTRODUCTION

Parkinson's disease (PD) is a debilitating neurological disorder with increasing prevalence in aging populations. The cardinal motor symptoms of PD arise from the progressive degeneration of nigrostriatal dopamine neurons and the resultant dopamine depletion in the dorsal striatum (Kalia and Lang 2015; McGregor and Nelson 2019). This causes imbalance in the excitability of the direct and indirect striatal projection pathways within the basal ganglia leading to inadequate activation of the motor cortex and impaired motor functions. The underlying pathological processes of PD are incompletely understood, but they seem to be associated with neuroinflammation and the presence of intracellular protein inclusions called Lewy bodies.

There is no cure for PD. The current treatments provide symptomatic relief mainly to the motor symptoms by increasing dopaminergic activity within the dorsal striatum (Armstrong and Okun 2020). With the progression of the disease, however, the therapies gradually lose their effects and start to be accompanied by troublesome adverse effects such as motor fluctuations, dyskinesias and psychiatric symptoms. The dopaminergic treatments have minor effects on disabling non-motor symptoms of PD and, importantly, they are unable to halt the neurodegenerative processes underlying the disease. Thus, a disease-modifying therapy remains an urgent unmet medical need for PD.

Our brain has an innate capacity to reorganize its structure, neuronal connections and functions in response to intrinsic and extrinsic stimuli (Cramer et al. 2011). These dynamic adaptations, commonly called neuroplasticity, have a pivotal role in the proper function of the nervous system. Neurotrophic factors (NTFs) are small secreted proteins that regulate almost all aspects of neuroplasticity including neurogenesis, neuronal development and maintenance and, importantly, the survival and recovery of neurons (Paratcha and Ledda 2008). Therefore, NTFs serve as promising candidates for developing disease-modifying therapies for neurodegenerative disorders such as PD.

There are four major families of NTFs, two of which, GDNF family ligands and CDNF/MANF family of NTFs, are of special interest in this thesis. Glial cell line-derived neurotrophic factor (GDNF) is the founding member of GDNF family ligands and was identified in 1993 based on its survival-promoting effects on cultured midbrain dopamine neurons (Lin et al. 1993). Thereafter, intensive research efforts have uncovered the signaling mechanisms of GDNF as well as its robust survival promoting and regenerative effects on midbrain dopamine neurons both *in vitro* and in animal models of PD.

Cerebral dopamine neurotrophic factor (CDNF) and mesencephalic astrocyte-derived neurotrophic factor (MANF) form structurally and functionally distinct family of NTFs (Lindholm et al. 2007; Petrova et al. 2003). They have also shown strong neurotrophic properties on midbrain dopamine neurons promoting their survival and repair *in vitro* and in animal models of PD. Their precise mechanism of action, however, has remained undetermined thus far. Being endoplasmic reticulum (ER)-resident proteins, CDNF and MANF have been suggested to regulate ER stress and

subsequent unfolded protein response (UPR). As inflammation and ER stress responses are closely linked with each other, their neuroprotective effects may be due to regulation of neuroinflammation in the brain.

Following promising outcomes in preclinical experiments, intracranial GDNF application proceeded into clinical trials with PD patients. These trials suggest a favorable safety profile for the NTF therapies together with some promising efficacy outcomes, yet none of the randomized, placebo-controlled trials met their primary endpoints (Nutt et al. 2003; Lang et al. 2006; Whone et al. 2019). The varying success in the clinical trials has raised questions about the optimal dosing paradigm and protein therapy related challenges. NTFs are, for example, unable to cross the blood-brain barrier (BBB), and thus require intracranial delivery increasing treatment-related risks and costs. A small molecule that mimics the effects of NTFs on degenerating dopamine neurons and is suitable for systemic administration would be an attractive drug candidate for PD.

In the present study, we pursued new insights into the biological effects of intrastriatally injected CDNF and MANF on dopamine synthesis, release and metabolism in the normal rat brain in order to gain better understanding of their therapeutic applicability for degenerative brain diseases. We also further elucidated the spreading properties of CDNF in the rat brain after intranigral injection which is highly relevant information in terms of finding the optimal sites of administration. Lastly, we investigated two novel small molecule RET agonists, their signaling properties and neuroprotective effects on nigrostriatal dopamine neurons in a neurotoxin-induced rat model of PD. These agonists serve as promising lead compounds that may open avenues in the search of a BBB-penetrating neurotrophic therapy for PD.

The literature review will first give an overview of neuroplasticity and NTFs, their structure, expression and signaling mechanisms, focusing on GDNF family ligands, CDNF/MANF family of NTFs and NTF mimetics. Then, the organization and functions of the basal ganglia circuitry will be reviewed together with dopamine projection pathways, modulatory effects and lifecycle in the brain. Finally, PD and preclinical and clinical studies of NTFs in PD will be described.

2 REVIEW OF THE LITERATURE

2.1 Neuroplasticity and neurotrophic therapies

In this thesis, neurotrophic therapies refer to a concept of utilizing endogenous NTFs or similarly acting small molecule compounds (i.e. NTF mimetics) to induce neuroplasticity changes in the central nervous system (CNS). Neurotrophic therapies are considered as potential disease-modifying treatments for various neurological disorders.

2.1.1 Neuroplasticity

The brain has traditionally been considered as a static organ, without turnover of neurons or capacity for repair after neuronal insults. However, it has a fundamental ability to reorganize its structure, connections and function in response to intrinsic and extrinsic stimuli (Cramer et al. 2011). These long-lasting and bidirectional alterations are of crucial importance for neuronal development and brain functions, e.g. learning. Developmental and environmental changes, diseases and therapeutic interventions induce adaptive neuroplastic changes which take place at many levels. During normal development, newborn neurons that are not functionally integrated into neural circuits are eliminated through selective apoptosis (Castrén and Hen 2013). It has been estimated, that up to 60% of the neurons originally generated die in most neuronal populations (Oppenheim 1991). This naturally occurring cell death is regulated by NTFs that promote the survival and maturation of neurons.

At cellular level, the morphology of mature neurons can be modified through arborization and pruning of axonal and dendritic branches and spines (Castrén and Hen 2013). The synapse-containing branches are more likely stabilized whereas spines lacking a synapse are readily pruned. The number of synapses is regulated dynamically through synaptogenesis and elimination of inactive synapses. At functional level, long-term potentiation (LTP) increases the synaptic strength, whereas long-term depression (LTD) suppresses it in inactive synapses. Active information transfer in LTP enhances the synaptic function and protects the synapse from pruning.

Growth factors, especially NTFs, have been shown to play a key role in regulating the dynamic adaptations in the neuronal connections and functions which are crucial for the proper function of the nervous system (Paratcha and Ledda 2008; Bothwell 2014; Lu et al. 2014; Levy et al. 2018). NTFs control almost all processes relating to the neuroplasticity including neural stem cell proliferation, migration and differentiation of neuroblasts, growth and survival of neurons, neurite outgrowth, formation of synapses and LTP. In addition, numerous transcriptional and epigenetic mechanisms regulate the expression of effector genes involved in neuroplasticity (Castrén and Hen 2013).

Brain resident innate immune cells, microglia, are also prominent players in the regulation of brain homeostasis and neuronal plasticity (Block and Hong 2005; Sierra et al. 2010; Kettenmann et al. 2013; Wake et al. 2013). Reactive microglia are primarily responsible for immune reactions in the

CNS in response to an infection or neuronal injury. Resting microglia, instead, are responsible for the surveillance and elimination of presynaptic terminals and dendritic spines in a process termed synaptic pruning. Microglia also release various substances such as adenosine triphosphate (ATP), NTFs and cytokines which regulate neuroinflammation, programmed cell death, neurogenesis, neuronal repair and synaptic connectivity. In addition, microglia phagocytose apoptotic cells and newborn neural progenitor cells. These functions contribute to the maturation, plasticity and homeostasis of neuronal circuits during development and adulthood.

New neurons are continuously supplied to the hippocampus and olfactory bulb in most mammals throughout life conferring plasticity to these neuronal circuits (Falk and Frisén 2005; Steiner et al. 2019). The newborn hippocampal neurons derive from local stem cells residing in the subgranular zone (SGZ) of the dentate gyrus, whereas neurons added to the olfactory bulb are derived from a neurogenic niche in the subventricular zone (SVZ) of the lateral ventricle wall from where they migrate along the rostral migratory stream (RMS). Innovative research strategies have demonstrated that substantial neurogenesis occurs also in the germinal areas of the adult human brain. The incorporation of 5-bromo-3'-deoxyuridine (BrdU), a synthetic nucleotide injected to cancer patients for diagnostic purposes, into the DNA of the dividing cells (Eriksson et al. 1998) or retrospective analysis of radioactive ^{14}C incorporated into the genomic DNA as a consequence of increased atmospheric levels of ^{14}C produced by nuclear weapon tests during the Cold War (Spalding et al. 2005) has enabled these research achievements. Measuring the level of nuclear-bomb-test-derived ^{14}C in the neuronal DNA Spalding and coworkers revealed extensive neurogenesis of hippocampal cells also in adult humans (Spalding et al. 2013). Similarly, retrospective ^{14}C-dating has revealed that a substantial number of newborn interneurons continuously integrates into the striatum (Ernst et al. 2014). In humans, neuroblasts arising from the SVZ provide a source for striatal neurons instead of migrating to the olfactory bulb via RMS.

2.1.2 Neurotrophic factors

NTFs are endogenous proteins secreted by a variety of tissues in the body (reviewed in Huang and Reichardt 2001; Airaksinen and Saarma 2002). Importantly, NTFs support the survival of neurons and help them to recover from injuries making them a promising therapeutic strategy not only for the management of neurodegenerative disorders, such as PD, Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS), but also for the treatment of neuronal traumas, e.g. spinal cord injury. There appears to be a shortage of NTFs in neurodegenerative diseases; for example in PD patients, decreased expression of GDNF, brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) have been reported in the surviving neurons of the substantia nigra (SN) (Mogi et al. 1999; Parain et al. 1999; Howells et al. 2000; Chauhan et al. 2001). Apart from their important effects on the development and maintenance of neurons, NTFs also exert several essential functions outside the nervous system in non-neural tissues.

2.1.2.1 Neurotrophic hypothesis

Mature neurons have highly polarized morphology; they consist of a cell body containing the nucleus and other organelles, dendrites receiving synaptic input from neighboring cells, and one

long axon, which ends in presynaptic terminals. According to the classical neurotrophic hypothesis the target tissue of neuronal innervation secretes limiting quantities of NTFs which are transported retrogradely along the axon to the cell body where they suppress apoptosis of the innervating neuron (Hamburger 1939, 1934; Hamburger and Levi-Montalcini 1949). In this way, NTF secretion by the target organ ensures the balance between the size of the target and the number of innervating neurons. During development, most neuronal populations are initially overproduced. Neuronal target fields, however, produce NTFs in amounts that are not sufficient for all neurons. The lack of target-derived NTFs drives the neurons without proper connections with the target organ into programmed cell death, thus, regulating the innervation density of the organ. The main caveat of the abovementioned hypothesis is that it has been demonstrated to hold true almost exclusively in the peripheral nervous system (PNS). In the CNS, the relationship between target-derived NTFs and neuronal survival appears to be more complex because neighboring cells also can provide trophic support to neurons through paracrine secretion and a neuron might be able to secrete NTFs itself through an autocrine loop (Cerchia 2006). In addition, the classical neurotrophic hypothesis has been broadened since some neuronal populations seem to depend on several different NTFs regulating concurrently or sequentially the target organ innervation (Davies 1996).

2.1.2.2 Characteristics and families of NTFs

Structurally mature NTFs proteins consist of approximately 100-160 amino acids and contain typically several conserved disulphide bridges between cysteine residues enabling closely related conformations within different NTF families (Ibáñez 1998; Airaksinen et al. 1999; Airaksinen and Saarma 2002; Lindholm and Saarma 2010; Bothwell 2014). Like most other secreted polypeptides, NTFs are synthetized and packaged into secretory vesicles in the rough ER. NTFs can be secreted from various neuronal and non-neuronal cells in the CNS and PNS. NTFs are commonly produced in the form of a precursor protein. The signal sequence is cleaved either intracellularly in secretory vesicles or by extracellular proteases producing mature NTFs. Mature NTFs usually form non-covalently associated dimers that bind to transmembrane receptor tyrosine kinases which in turn initiate intracellular signaling cascades resulting in trophic effects (Ibáñez 1998).

NTFs known today can be divided into four major families (Bothwell 2014; Ibáñez and Andressoo 2017; Lindahl et al. 2017):

1. neurotrophins including nerve growth factor (NGF), BDNF, neuorotrophin-3 (NT-3) and neuorotrophin-4 (NT-4)
2. GDNF family ligands (GFLs) including GDNF, neurturin (NRTN), artemin (ARTN), persephin (PSPN), and a distant member growth and differentiation factor-15 (GDF-15, also known as macrophage-inhibiting cytokine-1, MIC-1)
3. neurotrophic cytokines (=neurokines) including CNTF, cardiotrophin-1, leukemia inhibitory factor, neuropoietin, oncostatin M, cardiotrophin-like cytokine, interleukin 6 (IL-6), IL-11 and IL-27
4. CDNF/MANF family of NTFs.

Two of these families, GFLs and unconventional CDNF/MANF family of NTFs are in the focus of this thesis and will be discussed in more detail in the following chapters. Also, other trophic and growth factors, e.g. members of transforming growth factor β (TGF-β) superfamily, vascular endothelial growth factors, insulin-like growth factors and fibroblast growth factors (FGFs) are shown to have neurotrophic activities but those fall out of the scope of this thesis (Grothe and Timmer 2007; Zacchigna et al. 2008).

2.1.3 GDNF family ligands

GDNF was identified and isolated in 1993 based on its survival-promoting effects on ventral midbrain dopamine neurons (Lin et al. 1993). NRTN, ARTN and PSPN, the other three family members structurally similar to GDNF, were characterized within five years after the discovery of GDNF (Kotzbauer et al. 1996; Baloh et al. 1998; Milbrandt et al. 1998). GDF-15 is a distant member of the GFLs which functions as a peripheral signal downregulating food intake, energy expenditure and body weight in response to tissue damage and stress (Bootcov et al. 1997; Böttner et al. 1999; Hsiao et al. 2000). GFLs are distant members of TGF-β superfamily. Together with their receptors they form one of the major neurotrophic networks in the nervous system regulating the development, maintenance and function of a variety of neurons and glial cells (Ibáñez and Andressoo 2017). GFLs, their receptors and binding preferences are summarized in Figure 2.1.

2.1.3.1 Synthesis, structure and secretion of GFLs

GFLs are synthesized in the form of a precursor protein preproGFL (Lin et al. 1993; Lonka-Nevalaita et al. 2010). The pre-sequence guides GFLs to the ER for secretion. During secretion GFLs form disulfide-bonded homodimers and can be modified by N-linked glycosylation (Lin et al. 1993; Lonka-Nevalaita et al. 2010; Piccinini et al. 2013). Proteolytic cleavage of proGFLs into mature GFL proteins takes place extracellularly by furin, PACE4, PC5A, PC5B and PC7. After secretion GFLs bind to heparan-sulphate side chains of extracellular matrix proteoglycans which limits their diffusion in brain parenchyma and increases their local concentration.

GDNF, the founding member GFLs, is a ~20 kD, N-glycosylated protein consisting of 211 amino acids (Lin et al. 1993). The mature 134 amino acids long GDNF is formed after the cleavage of the pre- (19 amino acids) and pro- (58 amino acids) signaling sequences. The primary structure of GDNF contains seven conserved cysteine residues with the same relative spacing as in the other TGF-β superfamily members. The tertiary structure of GDNF is stabilized with three disulphide bridges that are formed between the cysteine residues. The one remaining cysteine connects two GDNF molecules with each other via a disulphide bridge forming GDNF homodimer.

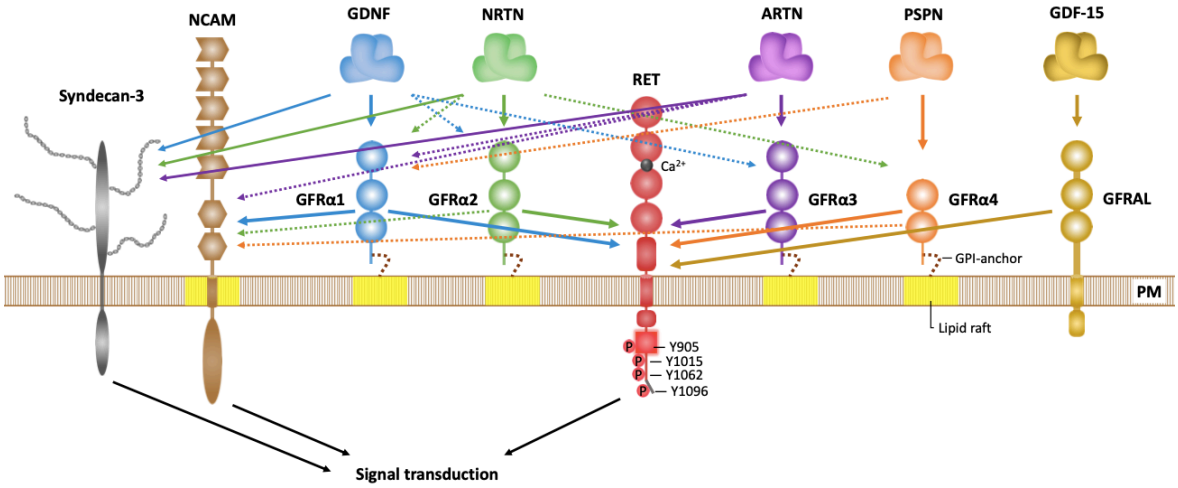

Figure 2.1. GDNF family ligands (GFLs) and their receptors. Glial cell line-derived neurotrophic factor (GDNF), neurturin (NRTN), artemin (ARTN), persephin (PSPN) and a distant member growth and differentiation factor-15 (GDF-15) bind preferentially to their cognate co-receptors GDNF family receptor α (GFRα) 1-4 and GDNF family receptor alpha-like (GFRAL), respectively, although cross-binding occurs too. Thereafter, ligand-receptor complex binds to the common transmembrane receptor tyrosine kinase RET or neural cell adhesion molecule (NCAM), which initiate intracellular signaling cascades. GFLs can also directly bind to and signal through syndecan-3. Glycosyl phosphatidylinositol (GPI) -anchor attaches GFRα receptors to the plasma membrane (PM) and localizes them into lipid rafts. Primary phosphotylated tyrosine residues of RET, which serve as docking sites for the intracellular adaptor proteins, and the Ca^{2+}-binding site in the middle of the four cadherin-like domains of the extracellular RET are depicted in the figure. High affinity binding is indicated with solid lines and low affinity binding with dashed lines. Figure drawn by the author, inspired by Kramer and Liss (2015).

2.1.3.2 RET-mediated signaling

Mature GFL homodimers mediate their biological effects via a multicomponent receptor complex consisting of GDNF family receptor α (GFRα) and receptor tyrosine kinase RET (rearranged during transfection) (Airaksinen and Saarma 2002). A GFL first binds to its cognate GFRα co-receptor which dimerizes. Subsequently, GFL-GFRα complex recruits RET as a signal transducing receptor and triggers its homodimerization and autophosphorylation of its intracellular tyrosine kinase domain. Apart from RET, two alternative signaling receptors have been identified: neural cell adhesion molecule (NCAM) (Chao et al. 2003; Paratcha et al. 2003) and syndecan-3 (Bespalov et al. 2011). A summary of the known GFL signaling mechanisms is illustrated in Figure 2.2.

RET was identified as the primary signaling receptor for GFLs in 1996 (Durbec et al. 1996a; Trupp et al. 1996; Vega et al. 1996; Worby et al. 1996). RET is a canonical single spanning transmembrane receptor tyrosine kinase. The extracellular region consists of four cadherin-like domains (CLD 1-4) with one Ca^{2+}-ion between CLD2 and CLD3 that is required for ligand binding to RET (Anders et al. 2001; Knowles et al. 2006). The cytoplasmic tyrosine kinase domain contains several tyrosine phosphorylation sites regulating the catalytic activities of RET (Myers et al. 1995; Tahira et al. 1990). In most cases, the phosphorylated residues are Tyr^{905}, Tyr^{1015}, Tyr^{1062} and Tyr^{1096} which serve as docking sites for signal-transducing adaptor proteins (Coulpier et al. 2002; Arighi et al. 2005). Alternative splicing of RET mRNA can produce three isoforms with varying length of the C-

terminal tail (RET9, RET43 and RET51). RET9 and RET51 are the major isoforms and highly conserved in different species of vertebrates (Carter et al. 2001).

Phosphorylation of the intracellular tyrosine residues of RET activates downstream signaling cascades including Ras/mitogen activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/Akt, phospholipase C-γ (PLCγ) and c-Jun N-terminal kinase (JNK) pathways (Takahashi 2001). Ras/MAPK pathway leads to the activation of transcription factor cAMP response element-binding protein (CREB) and regulates cellular proliferation, growth, differentiation, survival and neuritogenesis (Hayashi et al. 2000). PI3K/Akt pathway activates transcription factor NFκB and is primarily responsible for cellular survival, growth, migration and proliferation.

RET is unable to bind GFLs in absence of the ligand-binding co-receptor GFRα (Jing et al. 1996; Treanor et al. 1996; Paratcha and Ledda 2008). The GFRα:s are a family of four glycosyl phosphatidylinositol (GPI) -anchored extracellular proteins, GFRα1-4, that serve as preferential receptors for GDNF, NRTN, ARTN and PSPN, respectively (Jing et al. 1996; Treanor et al. 1996; Baloh et al. 1997; Buj-Bello et al. 1997; Jing et al. 1997; Klein et al. 1997; Sanicola et al. 1997; Suvanto et al. 1997; Baloh et al. 1998; Naveilhan et al. 1998; Thompson et al. 1998; Trupp et al. 1998; Widenfalk et al. 1998; Worby et al. 1998; Masure et al. 2000). In addition, GDNF family receptor alpha-like (GFRAL) was recently discovered as a co-receptor for GDF-15 (Emmerson et al. 2017; Hsu et al. 2017; Mullican et al. 2017; Yang et al. 2017). It mediates GDF-15–GFRAL signaling via RET in the same way as the other GFL–GFRα complexes. Apart from the high-affinity binding to the cognate co-receptors, some low-affinity cross-reactivities between different GFLs and GFRα:s have been demonstrated *in vitro* as summarized in Figure 2.1 and Table 2.1.

The GPI-anchor links GFRα:s to the plasma membrane and localizes them into lipid rafts, special subdomains of the plasma membrane (Tansey et al. 2000; Paratcha et al. 2001; Paratcha and Ibáñez 2002; Tsui et al. 2015). These cholesterol and sphingolipid-rich microdomains accumulate signaling proteins and thereby increase their interactions with each other. During receptor activation in *cis*, GFL homodimer first binds with high affinity to one of the GPI-anchored GFRα receptors which dimerizes (Airaksinen and Saarma 2002; Jing et al. 1996; Treanor et al. 1996) (Figure 2.2.A). Subsequently, GFL-GFRα complex recruits RET into the lipid raft. The relocation of RET into the lipid rafts potentiates downstream signal transduction through the receptor complex (Tansey et al. 2000). Through phosphorylated Tyr^{1062} residue, for example, RET associates with the adaptor protein FRS2 inside the lipid rafts and with SHC outside the rafts (Paratcha et al. 2001). Inside the lipid rafts, FRS2 first recruits Grb2 and Sos proteins which then leads to the activation of Ras/MAPK pathway (Melillo et al. 2001). Outside the lipid rafts, SHC can recruit either Grb2 and Gab proteins leading to the activation of PI3K/Akt pathway, or Grb2 and Sos proteins activating Ras/MAPK pathway (Besset et al. 2000; Hayashi et al. 2000).

The GPI-anchor of GFRα receptor can also be cleaved by membrane-associated phospholipases or proteases which releases GFRα into the extracellular space and leads to the activation of RET in *trans* (Yu et al. 1998; Paratcha et al. 2001; Ledda et al. 2002). Soluble GFRα binds GFL in the extracellular space with high affinity after which the complex binds to and activates RET outside the lipid rafts where RET associates with SHC adaptor protein. Subsequently, RET is recruited into

the lipid raft where it activates FRS2. The fact that GFRα receptors are much more widely expressed than RET, together with their ability to activate RET in *trans*, suggest non-cell-autonomous functions for soluble GFRα during neuronal development and regeneration. Secretion of soluble GFRα by target tissues of RET-expressing neurons may act as a long-range cue that guides the growing axons.

It should be noted, that RET signaling is under negative control which regulates the magnitude and duration of RET activation. A prevalent mechanism to control the downregulation of RET is through ligand-induced ubiquitination which leads to proteasomal degradation of the active receptor (Carniti et al. 2003; Scott et al. 2005). In response to ligand-mediated activation, RET can also be internalized from the plasma membrane into early endosomes via clathrin-mediated endocytosis (Richardson et al. 2006; Richardson et al. 2012; Crupi et al. 2015). The endocytosed GFL-GFRα-RET complex contributes to the retrograde GFL signaling, but finally receptor internalization terminates the extracellular signal transduction.

2.1.3.3 NCAM-mediated signaling

GFLs can also signal through NCAM independently of RET (Paratcha et al. 2003) (Figure 2.2.B). NCAM plays an important role in neurodevelopment, regeneration and synaptic plasticity by mediating cell adhesion to other cells and components of the extracellular matrix. GFLs, but not other NTFs, can bind directly to NCAM via its third Ig domain (Paratcha et al. 2003; Sjöstrand et al. 2007; Nielsen et al. 2009). However, high-affinity binding of a GFL and downstream signaling through NCAM require the presence of GFRα co-receptor (Paratcha et al. 2003). Binding of GFL-GFRα complex to NCAM activates intracellular Src family kinase Fyn and focal adhesion kinase (FAK) which, in turn, activate MAP kinases and cellular responses such as neurite outgrowth of hippocampal, cortical and midbrain neurons, survival of cultured dopamine neurons and stimulation of Schwann cell migration (Chao et al. 2003; Paratcha et al. 2003; Iwase et al. 2005; Cao et al. 2008a; Nielsen et al. 2009). GDNF has been shown to function as a chemoattractant factor for neuronal precursors migrating along the RMS and NCAM-mediated signaling seems to play a key role in this guidance process (Paratcha et al. 2006). A recent study suggested that NCAM can function as an alternative receptor also for ARTN (Ilieva et al. 2019). ARTN was shown to induce neuritogenesis by binding directly to NCAM and activating NCAM-associated signaling pathways in primary cerebellar neuron cultures.

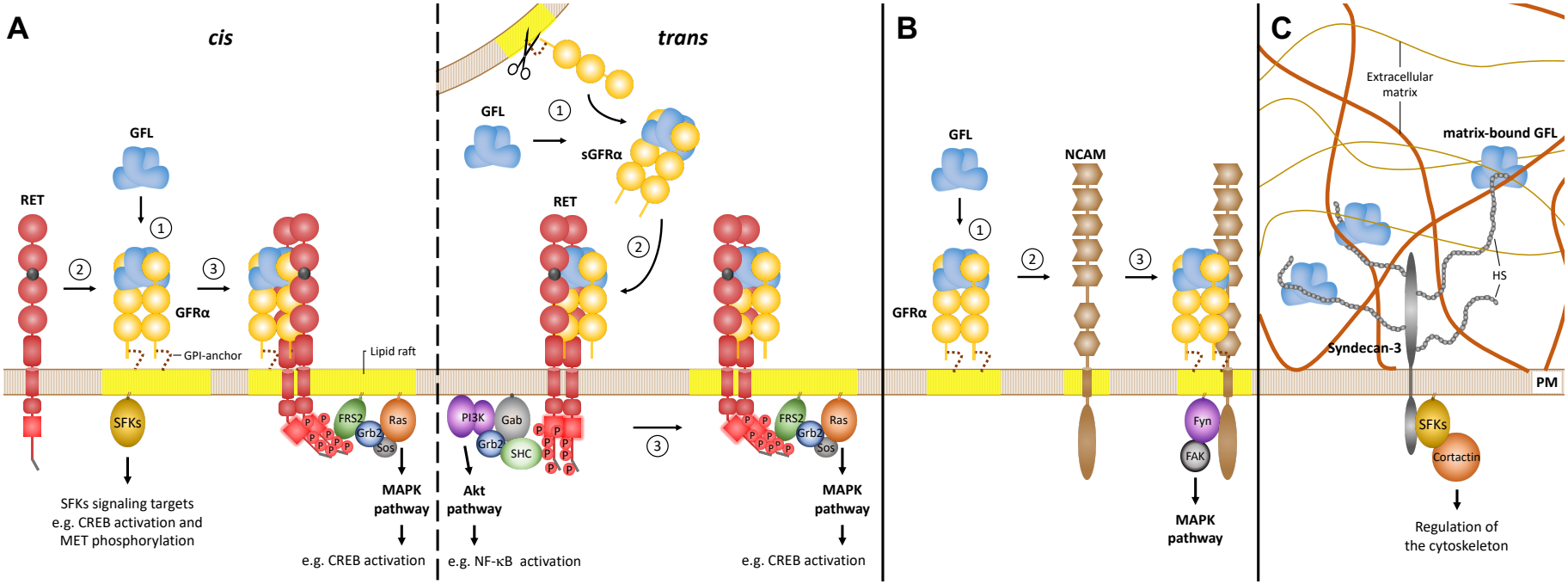

Figure 2.2. Signaling mechanisms of GDNF family ligands (GFLs). (**A**) Signaling through receptor tyrosine kinase RET can occur in *cis* or in *trans*. During activation of RET in *cis*, GFL homodimer first binds to a glycosyl phosphatidylinositol (GPI) -anchored GDNF family receptor α (GFRα) which dimerizes (1). Then, GFL-GFRα complex recruits two RET molecules into the lipid raft of plasma membrane (PM) (2). This triggers dimerization of RET and autophosphorylation of its intracellular tyrosine residues which serve as docking sites for intracellular adaptor proteins (3). In *trans* signaling, the GPI-anchor of GFRα is cleaved by membrane-associated phospholipases (scissors) which releases soluble GFRα (sGFRα) into extracellular space. GFL binds with high affinity to sGFRα (1). GFL-sGFRα complex activates RET outside of the lipid rafts (2). Subsequently, the receptor complex is recruited into the lipid raft (3). Inside the lipid rafts, phosphorylated RET associates with the adaptor protein FRS2 leading to the activation of Ras/MAPK pathway. Outside the rafts, RET interacts with SHC leading to the activation of PI3K/Akt pathway. GDNF can also activate intracellular Src family kinases (SFKs) signaling via GFRα1 independently of RET. (**B**) Neural cell adhesion molecule (NCAM) is an alternative signaling receptor for GFLs in cells lacking RET. GFL binds with high affinity to GFRα which dimerizes (1). Then, GFL-GFRα complex binds to transmembrane NCAM (2) leading to signal transduction via intracellular Src family kinase Fyn and focal adhesion kinase (FAK), and ultimately, the activation of MAP kinases (3). (**C**) Extracellular matrix-bound GFLs can also signal through a transmembrane heparan sulfate (HS) proteoglycan syndecan-3, independently of GFRα, RET or NCAM. Immobilized GFLs bind to the HS side chains of syndecan-3. The cytoplasmic domain activates intracellular SFKs and cortactin -mediated signaling cascades regulating the cytoskeleton. One syndecan-3 can bind several GFLs simultaneously. Figure drawn by the author.

2.1.3.4 Signaling via syndecan-3

The distribution of GDNF and NRTN in the brain parenchyma is restricted by binding to heparan sulfate proteoglycans of the extracellular matrix (Lin et al. 1994; Hamilton et al. 2001; Rickard et al. 2003; Piltonen et al. 2009; Bespalov et al. 2011). Bespalov and his colleagues showed that immobilized, extracellular matrix-bound GDNF, NRTN and ARTN can also signal through a novel receptor, the transmembrane heparan sulfate proteoglycan syndecan-3, that is expressed on neuronal cells (Bespalov et al. 2011) (Figure 2.2.C). Immobilized GFLs bind with high affinity to the heparan sulfate side chains of syndecan-3 which activates intracellular SFK-mediated signaling cascades. As syndecan-3 has several heparan sulfate chains, one syndecan-3 can simultaneously bind multiple GFL homodimers acting as a high affinity and high capacity receptor for GFLs. GFL–syndecan-3 interaction promotes hippocampal neurite outgrowth and migration of cortical γ-aminobutyric acid (GABA) containing neurons, and thus may play a distinctive role in the embryonic development of these brain areas. In addition to direct intracellular signal transduction, syndecan-3, and other heparan sulfate proteoglycans, may modulate conventional signaling through GFRα-RET or NCAM by concentrating and presenting diffusible GFLs to the receptors. GFLs may have a dual mode of action: as diffusible soluble proteins, they prefer signaling via conventional receptors GFRα-RET and NCAM, whereas extracellular matrix-bound GFLs seem to signal through syndecan-3 independently of GFRα, RET or NCAM.

2.1.3.5 Expression and functions of GFLs, GFRα:s and RET

Following the initial observation of the effects of GDNF on midbrain dopamine neurons, several studies have shown its neurotrophic actions in other neuronal populations of the CNS and PNS. For example, GDNF supports the survival of noradrenergic neurons of the locus coeruleus (Arenas et al. 1995), basal forebrain cholinergic neurons (Williams et al. 1996), facial and spinal cord motor neurons (Henderson et al. 1994; Oppenheim et al. 1995; Yan et al. 1995) and peripheral sympathetic, parasympathetic and sensory neurons (Buj-Bello et al. 1995; Ebendal et al. 1995; Trupp et al. 1995). The biological effects on such a broad spectrum of neuronal populations suggest widespread expression of GDNF and its receptors throughout the CNS, PNS and non-neuronal tissues.

Although all GFLs activate the same downstream signaling pathways through RET, the selectivity in their biological effects is thought to be due to differential expression patterns of GFLs and their cognate GFRα co-receptors. GFL, GFRα and RET expression levels have been investigated using Northern blotting, *in situ* hybridization, RT-PCR and immunohistochemical techniques. The neuronal and non-neuronal expression of GFLs, GFRα:s and RET in developing and adult rodents together with their physiological main functions are summarized in Table 2.1. In general, the levels and temporospatial expression patterns of GFLs are strictly regulated (Mogi et al. 2001). GFLs and their receptors are more prominently expressed during embryonic development as compared to adult animals (Golden et al. 1999). In the mature brain, GFRα receptors show wider expression than GFLs or RET (Nosrat et al. 1997; Trupp et al. 1997; Ortega-de San Luis and Pascual 2016). When RET and GFRα are expressed in the same tissue, such as in the SN or spinal cord, the receptors are able to interact in *cis*. However, the expression pattern of GFRα co-receptors does

not always match with that of RET which suggests in *trans* mode of interaction between the receptors or that GFLs convey RET-independent signaling via alternative receptors as described above.

Only GDNF and NRTN are expressed in the CNS of adult rodents which makes them to be of specific interest in regards of potential disease-modifying approaches for neurodegenerative diseases. Attempts to reveal the cellular basis of the relatively abundant expression of GDNF in postnatal striatum have shown that in the normal brain, neurons are the principal source of GDNF (Oo et al. 2005). More precisely, GDNF is mainly synthesized by striatal interneurons (Bizon et al. 1999; Hidalgo-Figueroa et al. 2012). The vast majority (approximately 95%) of the GDNF-expressing cells in the striatum are parvalbumin (PV) -positive GABAergic interneurons and the remaining 5% are either cholinergic or somatostatin-positive interneurons.

Upregulation of GDNF has been reported in several nigrostriatal injury models (Batchelor et al. 1999; Liberatore et al. 1997; Yurek and Fletcher-Turner 2002, 2001). Upon injury, glial cells become the predominant source of GDNF as well as other growth factors like NGF, NT-3 and FGF (Bresjanac and Antauer 2000; Nakagawa and Schwartz 2004; Nakagawa et al. 2005; Chen et al. 2006). The switch in the production of NTFs from neurons to glial cells may be part of the local mechanisms that aim to protect neurons and promote their regeneration. The cross-talk between damaged neurons and glia inducing the glial GDNF expression seems to be mediated via pro-inflammatory cytokines IL-1β, IL-6, interferon-γ (IFN-γ), TNF-α and TNF-β, FGF (Appel et al. 1997; Verity et al. 1998; Verity et al. 1999; Kuno et al. 2006; Saavedra et al. 2007) and endothelin-1 (Koyama et al. 2003a, 2003b).

Endogenous GDNF does not affect the normal embryonic development of brainstem noradrenergic and midbrain dopaminergic neurons (Moore et al. 1996; Sánchez et al. 1996). However, the physiological role of endogenous GDNF in the maintenance of normal adult brain catecholaminergic neurons has remained poorly studied because of neonatal mortality of GDNF full knockout mice due to renal and ENS agenesis. Interestingly, there is an ongoing debate whether GDNF is vital or dispensable for the survival of catecholaminergic neurons of the substantia nigra pars compacta (SNpc) and the locus coeruleus (Enterría-Morales et al. 2020; Kopra et al. 2015; Kumar et al. 2015; Pascual et al. 2008; Pascual and López-Barneo 2015). In adult mice, GDNF overexpression from the native locus seems to exert trophic effects on the nigrostriatal dopamine system and enhance its function (Kumar et al. 2015).

Table 2.1. GDNF family ligands (GFL), GDNF family ligand receptors (GFRα) and RET: expression in rodents and physiological main functions.

		Expression	Main functions	References
GFL	GDNF	**CNS:** cortical areas, STR, NAcc, Hc, thalamic nuclei, hypothalamic nuclei, OB, olfactory tubercle, septum, midbrain, PAG, cerebellum, pons (incl. pedunculopontine nucleus and locus coeruleus), medulla, spinal cord, pituitary gland, pineal gland*, wall of the 4th ventricle **PNS:** sensory neurons, sympathetic neurons, Schwann cells **Peripheral tissues:** skeletal muscle, gonads, prostate, lung, liver, adrenal gland, stomach, intestine, spleen, heart, thymus, thyroid, salivary glands, skin, kidney*, bone*, teeth*, ear*, nose*, tongue*, eye*, urogenital system*, digestive system*, limb buds*, cartilage*, blood*	• Originally discovered as a potent survival-promoting factor for embryonic midbrain dopamine neurons in culture • Essential for the prenatal development of ENS and kidneys • Essential for the development of peripheral sensory, sympathetic and parasympathetic neurons • Plays a crucial role in spermatogenesis • Supports the development and survival of spinal motoneurons, hindbrain noradrenergic neurons, midbrain dopamine neurons, basal forebrain cholinergic neurons and cerebellar Purkinje cells • May promote the insertion and stabilization of postsynaptic receptors in the neuromuscular junction • *GDNF*$^{-/-}$ genotype leads to perinatal death due to renal agenesis and the absence of enteric neurons	(Lin et al. 1993), (Scharr et al. 1993), (Henderson et al. 1994), (Springer et al. 1994), (Arenas et al. 1995), (Bowenkamp et al. 1995), (Buj-Bello et al. 1995), (Choi-Lundberg and Bohn 1995), (Ebendal et al. 1995), (Li et al. 1995), (Mount et al. 1995), (Oppenheim et al. 1995), (Springer et al. 1995), (Tomac et al. 1995), (Trupp et al. 1995), (Yan et al. 1995), (Hellmich et al. 1996), (Moore et al. 1996a), (Nosrat et al. 1996), (Pichel et al. 1996), (Sánchez et al. 1996), (Suvanto et al. 1996), (Vega et al. 1996), (Sainio et al. 1997), (Trupp et al. 1997), (Widenfalk et al. 1997), (Enomoto et al. 1998), (Golden et al. 1998), (Heuckeroth et al. 1998), (Fundin et al. 1999), (Golden et al. 1999), (Baudet et al. 2000), (Enomoto et al. 2000), (Garcès et al. 2000), (Meng et al. 2000), (Mikaels et al. 2000), (Worley et al. 2000), (Young et al. 2001), (Kramer et al. 2006), (Naughton et al. 2006), (Wang et al. 2010), (Savitt et al. 2012), (Ortega-de San Luis and Pascual 2016)
	Neurturin (NRTN)	**CNS:** cortical areas, STR, Hc, thalamic nuclei, hypothalamic nuclei, ventral midbrain, cerebellum, pituitary gland, septum*, brainstem nuclei*, pineal gland* **PNS:** sensory neurons, sympathetic neurons, retina **Peripheral tissues:** gonads, prostate, kidney, heart, bladder, urethra, skin, GI-tract, liver, lung, thymus, exocrine glands, skeletal muscle*, teeth*, digestive system*, sensory organs*	• Originally identified on the basis of survival-promoting effects on sympathetic neurons in culture • Essential for the normal development and survival of parasympathetic neurons • Essential for the proper development, maintenance and function of ENS • Contributes to food digestion by ensuring proper intestinal motility and secretion of pancreatic enzymes and saliva • Promotes the survival and neurite outgrowth of spinal motoneurons and embryonic basal forebrain cholinergic neurons and survival of sensory neurons and midbrain dopaminergic neurons • *NRTN*$^{-/-}$ mice are viable and fertile, no gross developmental defects except ptosis due of lack of parasympathetic innervation of the lacrimal gland	(Kotzbauer et al. 1996), (Klein et al. 1997), (Widenfalk et al. 1997), (Golden et al. 1998), (Heuckeroth et al. 1998), (Horger et al. 1998), (Bilak et al. 1999), (Forgie et al. 1999), (Fundin et al. 1999), (Golden et al. 1999), (Heuckeroth et al. 1999), (Jomary et al. 1999), (Rossi et al. 1999), (Taraviras et al. 1999), (Åkerud et al. 1999), (Baudet et al. 2000), (Enomoto et al. 2000), (Hiltunen et al. 2000), (Laurikainen et al. 2000), (Rossi et al. 2000), (Golden et al. 2003), (Rossi et al. 2003), (Cho et al. 2004b), (Mabe et al. 2006)
	Artemin (ARTN) *also termed enovin and neublastin*	**CNS:** *not detected* **PNS:** nerve roots of DRG*, immature Schwann cells*, sympathetic neurons*, along the routes of sympathetic neuroblast migration and along sympathetic axonal projections* **Peripheral tissues:** blood vessels (smooth muscle cells), esophagus*, stomach*, pancreas*	• Originally identified as the ligand for the orphan GFRα3–RET receptor • Vascular-derived NTF crucial for the migration and axonal outgrowth of sympathetic neuroblasts and development of target tissue sympathetic innervation • Promotes the survival of sensory and midbrain dopamine neurons • Modulates the sensitivity of sensory neurons to noxious stimuli • *ARTN*$^{-/-}$ mice are viable and fertile, no gross developmental defects except ptosis due of lack of sympathetic innervation to the superior tarsus muscle	(Baloh et al. 1998b), (Nishino et al. 1999), (Baudet et al. 2000), (Rosenblad et al. 2000), (Andres et al. 2001), (Enomoto et al. 2001), (Honma et al. 2002), (Wang et al. 2008), (McIlvried et al. 2010), (Nivlet et al. 2016)
	Persephin (PSPN)	**CNS:** cortical areas*, Hc*, STR*, diencephalon*, midbrain*, pons*, medulla*, cerebellum*, spinal cord*, astrocytes* **PNS:** sympathetic neurons (SCG)*, sensory neurons (DRG)*, sciatic nerve*, optic nerve*, motoneurons* **Peripheral tissues:** fat tissue, adrenal gland, heart, kidney, liver, skin, spleen*, skeletal muscle*, bone*, testicle* *In general, PSPN is expressed at very low levels in adult rodents*	• Originally discovered as the result of its homology to GDNF and NRTN • Regulates the function of thyroid C cells, their calcitonin production and bone formation in newborn and juvenile mice • May act as a circulating growth factor due to inability to bind heparan sulfate side chains of the extracellular matrix • Can modulate glutamate-mediated excitotoxicity in the CNS and supports the survival of motoneurons and sympathetic neurons • *In vitro,* promotes the survival and neurite outgrowth of embryonic midbrain dopamine and basal forebrain cholinergic neurons • *PSPN*$^{-/-}$ mice are viable and fertile, no gross developmental defects	(Enokido et al. 1998), (Heuckeroth et al. 1998), (Jaszai et al. 1998), (Milbrandt et al. 1998), (Tomac et al. 2002), (Åkerud et al. 2002), (Golden et al. 2003), (Lindfors et al. 2006), (Bespalov et al. 2011)

GFRα	GFRα1 *also termed GDNFR-α, TrnR1 and RETL1*	**CNS:** cortical areas, STR, NAcc, Hc, OB, thalamic nuclei, hypothalamic nuclei, OT, amygdala, septum, habenular nuclei, ventral pallidum, midbrain (incl. SNpc, SNr, VTA and dorsal Raphe nucleus), PAG, cerebellum, pons (incl. pedunculopontine nucleus and locus coeruleus), spinal cord **PNS:** sensory neurons, Schwann cells, ENS, motoneurons, sympathetic neurons*, parasympathetic neurons*, retina* **Peripheral tissues:** gonads, prostate, GI-tract, liver, kidney, bladder, urethra, heart, lung, spleen, digestive system*, skin*, bone*, teeth*, skeletal muscle*, inner ear*, endocrine glands*, salivary glands*	• Cognate co-receptor for GDNF • Shows low-affinity cross-reactivity with NRTN and ARTN *in vitro* • Association with NCAM potentiates high-affinity binding of GDNF to NCAM • Interaction with NCAM downregulates homophilic NCAM binding and cell adhesion • Ligand-induced cell adhesion molecule in the presence of GDNF • *GFRα1*$^{-/-}$ mice die early postnatally due to renal agenesis and deficits in the ENS	(Baloh et al. 1997), (Jing et al. 1997), (Klein et al. 1997), (Nosrat et al. 1997), (Sanicola et al. 1997), (Trupp et al. 1997), (Widenfalk et al. 1997), (Baloh et al. 1998b), (Cacalano et al. 1998), (Enomoto et al. 1998), (Glazner et al. 1998), (Golden et al. 1998), (Masure et al. 1998), (Yu et al. 1998b), (Fundin et al. 1999), (Golden et al. 1999), (Baudet et al. 2000), (Bennett et al. 2000), (Enomoto et al. 2000), (Hiltunen et al. 2000), (Mikaels et al. 2000), (Rossi et al. 2000), (Garcès et al. 2001), (Sarabi et al. 2001), (Paratcha et al. 2003), (Sarabi et al. 2003), (Cho et al. 2004a), (Ledda et al. 2007), (Omodaka et al. 2014), (Ortega-de San Luis and Pascual 2016)
	GFRα2 *also termed GDNFR-β, TrnR2, RETL2 and NTNR-α*	**CNS:** cortical areas, Hc, OB, thalamic nuclei, hypothalamic nuclei, OT, amygdala, septum, nucleus basalis of Meynert, midbrain (incl. SNpc, SNr, VTA and dorsal Raphe nucleus), PAG, cerebellum, brainstem (incl. locus coeruleus), spinal cord, pineal gland, pituitary gland, STR*, habenular nuclei* **PNS:** sensory neurons, sympathetic neurons, parasympathetic neurons, Schwann cells, ENS, retina, motoneurons* **Peripheral tissues:** gonads, GI-tract, heart, lung, spleen, thyroid gland, kidney, placenta, pancreas, urogenital system*, skin*, bone*, teeth*, skeletal muscle*, endocrine glands*, salivary glands*, sensory organs*	• Cognate co-receptor for NRTN • Shows low-affinity cross-reactivity with GDNF *in vitro* • Association with NCAM potentiates high-affinity binding of NRTN to NCAM *in vitro* • *GFRα2*$^{-/-}$ mice are viable and fertile, but have ptosis and grow poorly due to deficits in the enteric and parasympathetic nervous system	(Baloh et al. 1997), (Jing et al. 1997), (Klein et al. 1997), (Sanicola et al. 1997), (Widenfalk et al. 1997), (Golden et al. 1998), (Horger et al. 1998), (Masure et al. 1998), (Naveilhan et al. 1998), (Trupp et al. 1998), (Yu et al. 1998b), (Fundin et al. 1999), (Golden et al. 1999), (Jomary et al. 1999), (Rossi et al. 1999), (Baudet et al. 2000), (Bennett et al. 2000), (Enomoto et al. 2000), (Hiltunen et al. 2000), (Mikaels et al. 2000), (Rossi et al. 2000), (Garcès et al. 2001), (Paratcha et al. 2003), (Cho et al. 2004b), (Mabe et al. 2006), (Omodaka et al. 2014), (Ishida et al. 2016)
	GFRα3	**CNS:** OB, Hc, cerebellum, *expressed at low levels in the CNS* **PNS:** sensory neurons, sympathetic neurons, Schwann cells, sympathetic neuroblasts*, peripheral nerves*, retina* **Peripheral tissues:** epidermis, thymus, heart, lung, intestine, pancreas, spleen, ovary, kidney, adrenal medulla*, skeletal muscle*, salivary gland*, liver*	• Cognate co-receptor for ARTN • Shows low-affinity cross-reactivity with GDNF *in vitro* • *GFRα3*$^{-/-}$ mice are viable and fertile, no gross abnormalities other than ptosis	(Baloh et al. 1998a), (Masure et al. 1998), (Naveilhan et al. 1998), (Nomoto et al. 1998), (Trupp et al. 1998), (Widenfalk et al. 1998), (Worby et al. 1998), (Yu et al. 1998b), (Fundin et al. 1999), (Nishino et al. 1999), (Baudet et al. 2000), (Bennett et al. 2000), (Hiltunen et al. 2000), (Orozco et al. 2001), (Honma et al. 2002), (Omodaka et al. 2014), (Wong et al. 2015), (Nivlet et al. 2016)
	GFRα4	**CNS:** cortical areas, Hc, OB, habenular nuclei, ventral midbrain (incl. SNpc and VTA), cerebellum, spinal cord, pituitary gland **PNS:** sympathetic and parasympathetic ganglia **Peripheral tissues:** thyroid gland, parathyroid gland, adrenal medulla, heart, testicle	• Cognate co-receptor for PSPN • Shows low-affinity cross-reactivity with NRTN *in vitro* • Association with NCAM potentiates high-affinity binding of PSPN to NCAM *in vitro* • *GFRα4*$^{-/-}$ mice are viable and fertile, no gross developmental defects	(Enokido et al. 1998), (Lindahl et al. 2000), (Masure et al. 2000), (Åkerud et al. 2002), (Paratcha et al. 2003), (Lindfors et al. 2006)
RET		**CNS:** dopamine neurons of midbrain (incl. SN and VTA), serotonergic neurons of dorsal Raphe nucleus, cholinergic neurons of basal forebrain, OB, thalamic and hypothalamic nuclei, amygdala, septum, PAG, cerebellum, pons (incl. locus coeruleus), cranial and spinal motoneurons, *low levels in the STR* **PNS:** ENS, sympathetic neurons, parasympathetic neurons, sensory neurons, retina **Peripheral tissues:** testicle, salivary gland, GI-tract, adrenal medulla, thyroid gland, heart, lymphoid organs, kidney*, inner ear*, teeth*	• Transmembrane signaling receptor for GFL-GFRα complex • *Ret* gene originally discovered as an oncogene: gain-of-function mutations cause dominant cancer syndromes MEN2A, MEN2B and FMTC • Pivotal for the normal development and maintenance of ENS: loss-of-function mutations cause Hirschsprung's disease (intestinal obstruction/ megacolon) • Essential for kidney organogenesis and spermatogenesis • Essential for the development of sympathetic and parasympathetic neurons • Promotes the survival, axonal guidance and maturation of motoneurons • Regulates long-term maintenance of the nigrostriatal dopamine system • *RET*$^{-/-}$ mice die at birth due to renal agenesis and deficits in the ENS	(Takahashi et al. 1985), (Pachnis et al. 1993), (Schuchardt et al. 1994), (Tsuzuki et al. 1995), (Durbec et al. 1996b), (Schuchardt et al. 1996), (Trupp et al. 1996), (Nosrat et al. 1997), (Trupp et al. 1997), (Glazner et al. 1998), (Golden et al. 1998), (Yu et al. 1998b), (Golden et al. 1999), (Taraviras et al. 1999), (Bennett et al. 2000), (Enomoto et al. 2000), (Garcès et al. 2000), (Hiltunen et al. 2000), (Lindahl et al. 2000), (Enomoto et al. 2001), (Garcès et al. 2001), (Golden et al. 2003), (Jain et al. 2004), (Shakya et al. 2005), (Kramer et al. 2006), (Plaza-Menacho et al. 2006), (Kramer et al. 2007), (Mijatovic et al. 2007), (Baudet et al. 2008), (Jijiwa et al. 2008), (Uesaka et al. 2008), (The Human Protein Atlas 2020)

CNS, *central nervous system*; PNS, *peripheral nervous system*; ENS, *enteric nervous system*; DRG, dorsal root ganglia; SCG, superior cervical ganglia; STR, *striatum*; NAcc, *nucleus accumbens*; Hc, *hippocampus*; GI, *gastrointestinal*; OB, *olfactory bulb*; OT, *olfactory tubercle*; PAG, *periaqueductal gray*; SNpc, *substantia nigra pars compacta*; SNr, *substantia nigra pars reticulata*; VTA, *ventral tegmental area*; FMTC, *familial medullary thyroid carcinoma*; MEN2, *multiple endocrine neoplasia type 2*

* expression reported only during embryonic or early postnatal development, but not in adult rodent

2.1.4 CDNF/MANF family of neurotrophic factors

More recently discovered NTFs, MANF and its paralog CDNF are structurally and functionally distinct from the classical NTFs (Petrova et al. 2003; Lindholm et al. 2007; Lindahl et al. 2017). MANF (also known as arginine-rich, mutated in early stage tumors; ARMET) and CDNF (also known as ARMET-like 1; ARMETL1) are both present in vertebrates, but invertebrates, including *D. melanogaster* and *C. elegans*, express only one homologous protein that is more closely related to MANF (DmMANF) (Palgi et al. 2009). Together CDNF and MANF form an evolutionary conserved family of NTFs that shows classical neurotrophic properties such as promoting the survival and repair of midbrain dopamine neurons *in vitro* and in animal models of PD, protecting cardiac myocytes and cortical neurons against ischemia, and enhancing peripheral nerve recovery in a sciatic nerve transection model (Petrova et al. 2003; Lindholm et al. 2007; Tadimalla et al. 2008; Airavaara et al. 2009; Voutilainen et al. 2009; Airavaara et al. 2010; Voutilainen et al. 2011; Airavaara et al. 2012; Glembotski et al. 2012b; Cheng et al. 2013a; Cordero-Llana et al. 2015a; Liu et al. 2018; Zhang et al. 2018a; Zhang et al. 2018b).

During embryonic development, MANF signaling seems to be necessary for the proper neuronal differentiation, neuroblast migration and neurite outgrowth in the developing mouse brain (Tseng et al. 2018, 2017). In line, studies in *D. melanogaster* and *C. elegans* suggest an important role for DmMANF in the maturation and survival of dopamine neurons and in the maintenance of ER homeostasis (Palgi et al. 2009; Richman et al. 2018). In zebrafish, MANF knockdown causes a decrease in brain dopamine level and impairs the development of tyrosine hydroxylase (TH) expressing neurons (Chen et al. 2012). As for CDNF, knockout mice show age-dependent aberrant functions of the brain dopamine system such as slower dopamine reuptake via dopamine transporter (DAT) and increased amphetamine-induced dopamine release in the striatum (Lindahl et al. 2020). However, the number of dopamine neurons in the SN and dopamine level in the striatum remain unaltered. Interestingly, CDNF deficiency leads to age-dependent loss of enteric neurons in the submucosal plexus due to increased autophagy and neuronal degeneration. To date, the structure and expression pattern of CDNF and MANF are well-studied but their cell surface receptors and signaling pathways remain to be unraveled.

2.1.4.1 Structure and secretion of MANF and CDNF

CDNF and MANF are structurally unrelated to other NTFs. The amino acid sequences of mature human CDNF and MANF are 59% identical (Petrova et al. 2003; Lindholm et al. 2007; Lindahl et al. 2017). Their primary structure contains an N-terminal signal sequence (pre-sequence) that directs them to the ER during protein synthesis and allow access to the secretory pathway. Cleavage of the pre-sequence results in a mature protein that can be secreted via the canonical COPII-mediated ER-Golgi pathway (Apostolou et al. 2008; Oh-hashi et al. 2012; Norisada et al. 2016). Secreted CDNF and MANF seem to exert their trophic actions both in an autocrine and paracrine fashion but their binding to a transmembrane receptor has remained obscure until today (Apostolou et al. 2008; Lindahl et al. 2017). Although CDNF and MANF can be secreted, they are mostly retained intracellularly within the ER unlike classical NTFs that are exclusively secretory

proteins (Lindholm and Saarma 2010). In the ER, they seem to support protein folding and mitigate ER stress.

Human MANF is a glycosylated protein consisting of 179 amino acids and having a molecular mass of 20 kDA (Figure 2.3A) (Petrova et al. 2003). Its primary structure contains a 21-amino acid-long signal sequence which is cleaved to form 158 amino acids long mature protein. The secondary structure of the protein is dominated by α-helices and random coils and virtually lacks β-sheets. Human CDNF is 187 amino acids long, glycosylated protein with a calculated molecular mass of 18 kDa (Lindholm et al. 2007; Apostolou et al. 2008; Sun et al. 2011). CDNF has a signal sequence of 26 amino acids, the cleavage of which forms a 161-amino acid-long mature protein. Similarly to MANF, the secondary structure of CDNF contains mostly α-helices. A characteristic feature of the primary structure of CDNF and MANF is eight conserved cysteine residues (Lindholm et al. 2007; Petrova et al. 2003). Four disulphide bridges are formed between the cysteine residues stabilizing the tertiary structure of the mature proteins.

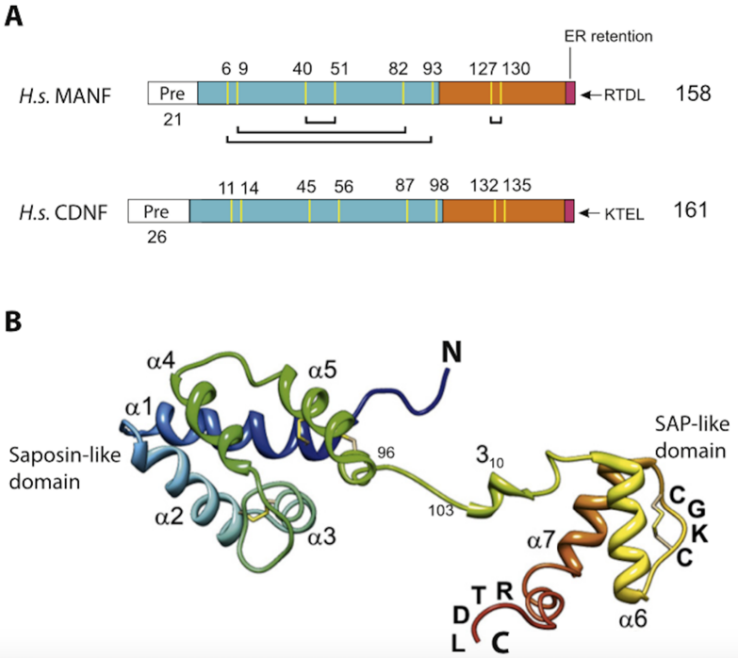

Figure 2.3. Schematic structure of human MANF and CDNF. (**A**) Schematic picture of the primary structure of human MANF and CDNF. The N-terminal (saposin-like) domain of MANF and CDNF is indicated in blue, and the C-terminal (SAP-like) domain in orange. Vertical yellow bars depict conserved cysteine residues, and they are numbered according to the sequence of mature protein. The formation of disulphide bridges is shown as black connecting lines below the sequence of MANF. The ER retention signals of MANF and CDNF (RTDL and KTEL, respectively) are marked with pink. (**B**) Solution structure of human MANF. MANF has two domains: saposin-like N-terminal domain containing five α-helices (α1-α5) and 3_{10} helix in the linker region, and SAP-like C-terminal domain containing two α-helices (α6 and α7). Yellow sticks show the formation of disulphide bridges in the tertiary structure. The CXXC motif (^{127}CKGC^{130}) and ER retention signal (^{155}RTDL^{158}) are indicated in the C-terminal domain. N - amino-terminus; C - carboxy-terminus. Adapted with permission from Elsevier: Neurobiology of Disease, Lindahl et al. (2017), © Elsevier 2016.

The tertiary structures of CDNF and MANF closely resemble each other. They consist of two domains connected to each other with a flexible linker region (Lindholm et al. 2007; Parkash et al. 2009; Hellman et al. 2011; Latge et al. 2015). N-terminal domain has a globular conformation containing five α-helices (α1-α5), 3_{10} helix in the linker region and a hydrophobic core. Three out of four disulphide bridges are located in the N-terminal domain as depicted in Figure 2.3B. C-terminal domain is partially unstructured; it contains two α-helices (α6 and α7) and one internal disulphide bridge between the two cystein recidues in CXXC motif (^{132}CRAC^{135} in CDNF and ^{127}CKGC^{130} in MANF). This CXXC motif in the loop connecting the helices α6 and α7 seems to be important for the functionality of the proteins. Mätlik and coworkers showed that mutating the CKGC motif abolishes the survival-promoting activity of MANF when overexpressed intracellularly in cultured sympathetic or sensory neurons, or when applied extracellularly in a rat model of focal cerebral ischemia (Mätlik et al. 2015). At the very end of the C-terminus, CDNF and MANF have conserved four amino acid sequences (^{158}KTEL^{161} in CDNF and ^{155}RTDL^{158} in MANF) which resemble the canonical ER retention signal KDEL (Glembotski et al. 2012; Henderson et al. 2013; Oh-hashi et al. 2012; Raykhel et al. 2007). Human has three KDEL receptors (KDELR1-3) that function in the Golgi. KDELRs recognize KDEL-like sequences in the C-terminus of proteins and mediate their retrograde trafficking from the Golgi back to the ER. Henderson and colleagues suggested that KDELRs, accumulated to the plasma membrane in ER-stressed cells, could mediate the cell surface binding of MANF (possibly CDNF too) through the interaction between the C-terminal RTDL sequence and KDELRs (Henderson et al. 2013).

MANF retention to the ER is also dependent on another ER resident protein, glucose-regulated protein 78 kDa (GRP78; also known as binding immunoglobulin protein, BiP), the expression of which closely resembles the expression of MANF in mouse tissues (Mizobuchi et al. 2007; Glembotski et al. 2012; Oh-hashi et al. 2012). MANF interacts directly with GRP78 and the interaction has been shown to be calcium dependent. Depletion of ER calcium storages e.g. by thapsigargin, an inhibitor of SERCA (sarco/endoplasmic reticulum calcium transporting ATPase), triggers MANF secretion independently of the RTDL sequence. Taken together, if the ER calcium concentration is normal, both GRP78 and KDELR seem to participate in retaining MANF in the ER. Upon ER calcium depletion, MANF dissociates from GRP78 leading to its increased secretion although the interaction with KDELR remains unaffected. Thus, the RTDL sequence is anticipated to function as a weak retention motif fine-tuning the secretion of MANF. Overexpression of GRP78 and KDELR1 in HEK293 cells similarly decreased the secretion of MANF and CDNF suggesting that the mechanisms regulating the secretion of CDNF and MANF are fundamentally similar (Glembotski et al. 2012; Oh-hashi et al. 2012; Henderson et al. 2013; Norisada et al. 2016).

2.1.4.2 Expression of MANF and CDNF

MANF is widely expressed both in neuronal and non-neuronal tissues starting from early stages of embryonic development until adulthood (Lindholm et al. 2008; Wang et al. 2014). Immunohistochemical studies show that in the rodent brain the expression of MANF is mainly neuronal (Lindholm et al. 2008; Shen et al. 2012; Wang et al. 2014). In the CNS of the adult mouse, MANF expression is abundant in several brain regions including the olfactory bulb, cortical areas, hippocampus, several hypothalamic and thalamic nuclei, pons, medulla, cerebellar Purkinje cells,

spinal cord and neural progenitors of the SVZ (Lindholm et al. 2008; Wang et al. 2014; Tseng et al. 2018). In the striatum and SN, only low to intermediate levels of MANF were detected both from the adult brain and during embryonic development. In the SNpc, MANF immunofluorescency co-localized with the TH-immunoreactive (ir) cells indicating MANF expression in dopamine neurons (Lindholm et al. 2008). However, MANF-positive neurons were more prevalent in the SN pars reticulata than in pars compacta. Wang and colleagues suggested that in the rat brain MANF expression is developmentally regulated with the expression being highest within the first two weeks after birth and decreasing upon aging (Wang et al. 2014).

Apart from the CNS, MANF is broadly expressed in peripheral tissues of adult and embryonic mice (Mizobuchi et al. 2007; Lindholm et al. 2008; Lindahl et al. 2014). The expression is ample especially in secretory cells and tissues, including choroid plexus, pancreatic exocrine acinar cells and endocrine β cells, salivary glands, liver and testis, suggesting an important role of MANF in regulating protein homeostasis in cells with a high rate of protein production. Lower levels of MANF are detected in the lung, skeletal muscles, kidney and heart (Lindholm et al. 2008). The indispensable role of MANF for the survival and normal function of pancreas was shown in MANF knockout mice (Lindahl et al. 2014). MANF deficiency caused progressive loss of functional β cells and chronic UPR activation in pancreatic islets, resulting in insufficient insulin secretion, increased blood glucose level and diabetic phenotype. Importantly, MANF is widely expressed in spleen and different types of immune cells (Cheng et al. 2014; Liu et al. 2015b; Neves et al. 2016). For example, MANF levels in peripheral leukocytes and synovial tissues are highly up-regulated in response to arthritis or other inflammatory diseases (Chen et al. 2015). Recently, MANF expression has also been detected in human blood serum (Galli et al. 2016) as well as in rodent and human optic nerves and retina where it promotes the survival of retinal ganglion and photoreceptor cells (Gao et al. 2017a; Gao et al. 2017b; Lu et al. 2018).

Similarly to MANF, CDNF transcripts are widely expressed both in the human and mouse brain as well as in peripheral non-neuronal tissues (Lindholm et al. 2007). The expression levels of CDNF, however, appear to be generally lower as compared to MANF. In immunohistochemical analyses, CDNF immunoreactivity was detected in somas where it co-localized with neuronal markers NeuN and Tuj1 indicating that the expression of CDNF is neuronal (Lindholm et al. 2007; Zhou et al. 2016). In addition, CDNF was shown to co-localize with glial fibrillary acidic protein (GFAP), a marker for astrocytes, in rat primary hippocampal cell culture. In the adult mouse brain, CDNF-positive cells were observed in the brain stem, including locus coeruleus, in the cortical layers II-VI, in CA1 and CA3 pyramidal regions and dentate gyrus of the hippocampus and in the Purkinje cells of the cerebellum (Lindholm et al. 2007). In the SN, however, CDNF immunoreactivity was detected only in solitary cells which did not include TH-positive dopamine neurons. CDNF staining was weak also in the striatum. In postnatal P1 and P10 mouse brains, CDNF signal was detected in the hippocampus, thalamus, striatum and SN. Relatively high CDNF levels were detected in the adult mouse heart, skeletal muscles and testis. Overall, the expression pattern of CDNF and MANF seems to partly overlap but the intensity of their expression differs.

2.1.4.3 Two proposed mechanisms of action

As described above, CDNF and MANF possess a two-domain protein structure (Parkash et al. 2009; Hoseki et al. 2010; Hellman et al. 2011; Latge et al. 2015). The domains are connected via a short polypeptide linker which allows them to move flexibly in relation to each other. The independent orientation of the domains may be an important feature in the mechanism of action of CDNF and MANF since the domains seem to exert distinct functions.

Anti-apoptotic effect of MANF and CDNF

The C-terminal domain of CDNF and MANF is homologous to the SAP (SAF-A/B, Acinus, and PIAS) domain of Ku70 (C-Ku70) (Hellman et al. 2011; Hoseki et al. 2010; Latge et al. 2015; Parkash et al. 2009). Ku70 inhibits the cytoplasmic pro-apoptotic Bax (Bcl-2-associated X protein) via its SAP domain thereby preventing apoptosis (Sawada et al. 2003; Wolter et al. 1997). During apoptosis, Ku70 dissociates from Bax which is translocated into mitochondria where it promotes release of cytochrome *c* into the cytosol. Cytochrome *c*, in turn, activates caspases resulting in cell death (Green 2000). Owing to the structural homology with the SAP domain of Ku70, the C-terminal domain of CDNF or MANF may prevent apoptosis by regulating Bax and subsequent caspase activation (Hellman et al. 2011; Mei and Niu 2014). Direct interaction between CDNF or MANF and Bax, however, has not been detected suggesting that the neuroprotective activity against Bax-dependent apoptosis is not mediated by a direct interaction of the proteins (Mätlik et al. 2015).

The N-terminal domain of CDNF and MANF has a homologous structure with saposin-like proteins (SAPLIPs), a diverse family of lipid and membrane -interacting proteins with various cellular functions (Parkash et al. 2009; Hoseki et al. 2010; Hellman et al. 2011; Latgé et al. 2013; Latge et al. 2015). SAPLIPs are globular proteins containing four or five α-helices and two or three disulphide bridges (Bruhn 2005). Structurally N-CDNF and N-MANF are closest to the membrane-lytic proteins granulysin and NK-lysin which contain several positively charged amino acid residues responsible for their binding to the negatively charged phospholipid heads of biological membranes (Parkash et al. 2009). Similarly, positively charged lysine and arginine residues on the surface of helices α3, α4 and α5 in N-CDNF and N-MANF may increase the membrane binding properties of the proteins. Lipid-binding could potentially mediate the initial cellular interaction and internalization of the proteins. Nevertheless, in spite of the structural propensity, lipid interactions of CDNF and MANF have not been demonstrated so far (Lindahl et al. 2017).

Regulation of ER stress by MANF and CDNF

Different cellular perturbations, for example the expression of mutated proteins or viral infections, can lead to the abnormal accumulation of unfolded or misfolded proteins in the lumen of ER. The misfolded proteins alter the function of ER and cause disturbances in cellular proteostasis referred to as ER stress (reviewed in Ron and Walter 2007; Dufey et al. 2014). In order to cope with protein folding alterations, ER stress triggers the activation of conserved cellular signaling cascades collectively known as UPR. UPR leads to adaptive responses that suppress protein translation and translocation into the ER, enhance protein folding capacity by increasing the production of molecular chaperones and degrading misfolded proteins. Altogether, these events aim to

attenuate unfolded protein load in the ER and restore the homeostasis. UPR related cytosolic signaling cascades are activated by dissociation of GRP78 from the luminal domain of three different ER transmembrane sensor proteins: inositol-requiring enzyme 1 (IRE1), protein kinase RNA-like ER kinase (PERK) and activating transcription factor 6 (ATF6). If ER stress becomes prolonged or too intense to be resolved with adaptive changes, UPR triggers apoptotic cell death mechanisms. ER stress and UPR play an important part in the pathophysiology of many neurodegenerative diseases, such as PD or AD, which are characterized by the aggregation of misfolded proteins in the brain (Hetz and Saxena 2017).

CDNF and MANF have been shown to localize in the ER suggesting their function as ER stress and protein homeostasis regulating proteins (Apostolou et al. 2008; Cheng et al. 2013b; Mizobuchi et al. 2007; Tadimalla et al. 2008; Voutilainen et al. 2015). The promoter region of *Manf* gene contains two ER stress-responsive elements (ERSE I and ERSE II) which are recognized by ER stress-inducible transcription factors activated ATF6 and spliced X-Box-Binding Protein 1 (XBP1s) enhancing MANF transcription (Lee et al. 2003; Yamamoto et al. 2004; Mizobuchi et al. 2007; Oh-Hashi et al. 2013; Wang et al. 2018a). On the contrary, *Cdnf* gene has not been reported to contain such elements. MANF and CDNF expression levels are upregulated upon ER stress in various cell types *in vitro* and *in vivo* (Apostolou et al. 2008; Glembotski et al. 2012; Hartley et al. 2013; Mizobuchi et al. 2007; Tadimalla et al. 2008; Zhang et al. 2018). In physiological conditions, MANF and CDNF mostly localize to the luminal side of the ER, whereas under ER stress their secretion is enhanced which is an unconventional response for an ER stress-induced protein.

The ER stress regulation of MANF and CDNF is proposed to be mediated through GRP78 and its downstream UPR signaling pathways (Lindahl et al. 2017). The hypothetical mechanism of MANF in the regulation of UPR is depicted in Figure 2.4. In a rat model of focal cerebral ischemia, for example, intraventricularly administered MANF was able to rescue the neuronal loss in the hippocampus and cortex and downregulate UPR by repressing the elevated levels of GRP78, phosphorylated IRE1 and cleaved caspase-3 (Yang et al. 2014). Similarly, exogenously administered CDNF protected against Aβ-induced synaptotoxicity by suppressing the increase of GRP78, phosphorylated JNK and cleaved caspase-3 in primary hippocampal cells (Zhou et al. 2016). In knockout mice, the lack of MANF led to chronic UPR activation in pancreatic islets and significant upregulation of GRP78, ATF4, CHOP and ATF6 (Lindahl et al. 2014). Recent findings by Yan and coworkers show that MANF binds to the nucleotide-binding domain of GRP78 and inhibits nucleotide exchange on GRP78 (Yan et al. 2019). Thus, MANF seems to promote protein folding in the ER by stabilizing GRP78–substrate interactions.

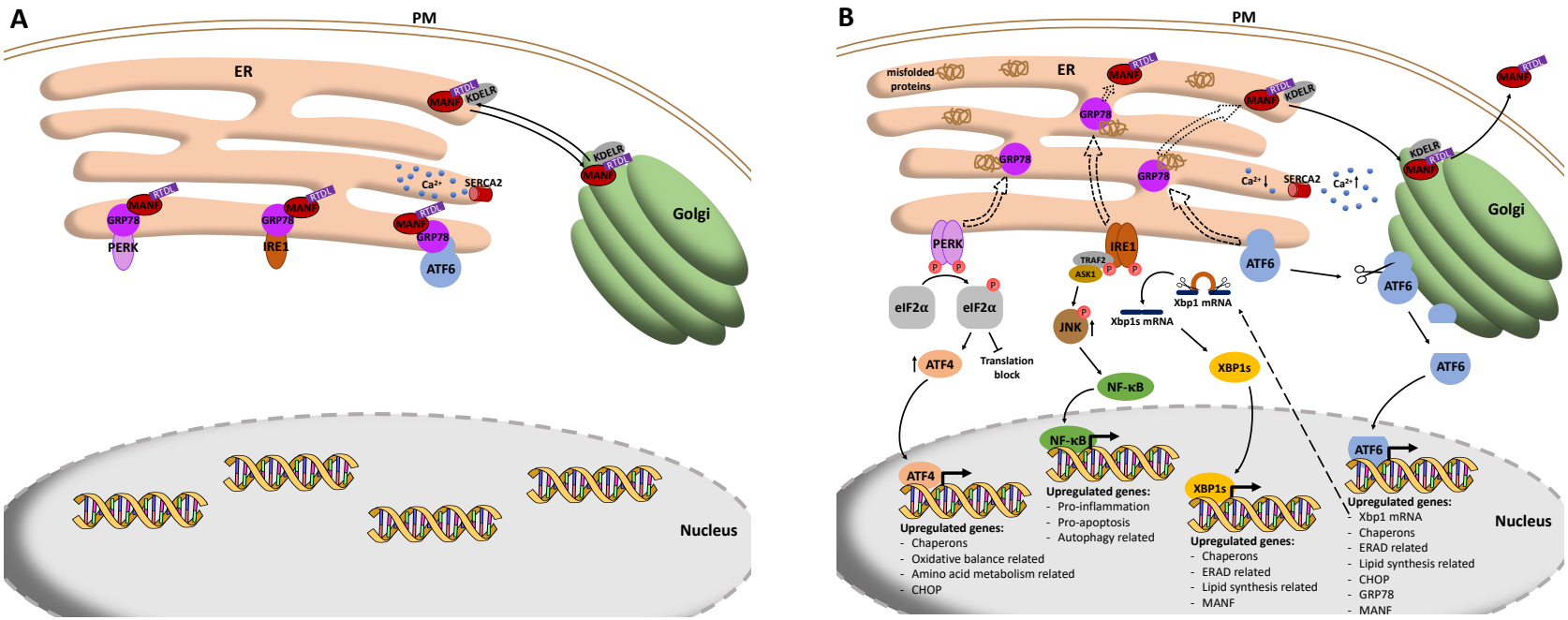

Figure 2.4. Proposed mechanism for unfolded protein response (UPR) regulation by MANF. (**A**) In the absence of endoplasmic reticulum (ER) stress, binding of glucose-regulated protein 78 kDa (GRP78) to the luminal domain of three ER transmembrane sensor proteins, activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1) and protein kinase RNA-like ER kinase (PERK), keeps them inactive. MANF is suggested to bind GRP78 in a Ca^{2+} dependent manner helping to keep the UPR sensor proteins inactive. KDEL receptor (KDELR) retains MANF in the ER via the C-terminal RTDL sequence. MANF may also be retrieved from the Golgi back to the ER by KDELR. (**B**) Upon ER stress, depletion of Ca^{2+} from the ER leads to dissociation of MANF from GRP78 and its secretion to the extracellular space, possibly due to increased competition in KDELR-mediated retention. GRP78 dissociates from ATF6, IRE1 and PERK and binds to accumulating misfolded proteins in the ER. This activates UPR related cytosolic signaling cascades. ATF6 is translocated to the Golgi, where it gets cleaved and activated. Active ATF6 moves into the nucleus, where it functions as a transcription factor for genes involved in protein folding, ER-associated protein degradation (ERAD) and lipid synthesis. It also upregulates the expression of GRP78, MANF, pro-apoptotic transcription factor C/EBP homologous protein (CHOP) and Xbp1 mRNA. Dissociation of GRP78 allows IRE1 to be dimerized and autophosphorylated. Endoribonuclease activity of the cytoplasmic domain of IRE1 removes an intron from Xbp1 mRNA. Spliced Xbp1s mRNA is then translated to XBP1s transcription factor, which upregulates genes for chaperons, ERAD, lipid synthesis and MANF. In addition, IRE1 can reduce translational workload by degrading ER associated mRNAs. Chronic activation of IRE1 can recruit TRAF2 and ASK1, which phosphorylates c-Jun N-terminal kinase (JNK) and leads to the nuclear translocation of NFκB. NFκB upregulates genes of inflammatory response, autophagy regulation and apoptosis. Dimerization and autophosphorylation of PERK followed by phosphorylation of translation initiation factor eIF2α leads to general inhibition of protein synthesis and increase in the translation of transcription factor ATF4. ATF4 induces genes related to protein folding, amino acid metabolism and restoring oxidative balance, as well as CHOP. PM – plasma membrane; SERCA2 – sarco/endoplasmic reticulum Ca^{2+}-ATPase2. Figure drawn by the author, inspired by Lindahl et al. (2017), Kim et al. (2017) and Danilova and Lindahl (2018).

Regulation of inflammation by MANF and CDNF

Inflammation and ER stress are closely linked with each other. In many pathologies, such as neurodegenerative diseases, all three ER stress-induced UPR sensor proteins (i.e. IRE1, PERK and ATF6) participate in mediating inflammatory processes as reviewed by (Zhang and Kaufman 2008; Cao et al. 2016; Sprenkle et al. 2017). ER stress and immune cell activation share many common regulators, e.g. NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), JNK and p38, forming a vicious cycle of the two signaling pathways that may exacerbate their pro-apoptotic effects. As a consequence, the neuroprotective and regenerative properties of CDNF and MANF may ultimately be due to regulation of inflammatory responses in the CNS (Zhao et al. 2014; Chen et al. 2015; Neves et al. 2016). Immune cell activation occurs at early stages of tissue reparation and its regulation is crucial to regenerative success (Sousa-Victor et al. 2018). The precise mechanisms through which CDNF and MANF can affect neuroinflammation still remain undetermined, but the studies published to date support the notion that they can reduce the secretion of pro-inflammatory cytokines and promote the pro-regenerative activation of immune cells.

MANF has been shown to exert immune modulatory functions that bias immune cells toward an anti-inflammatory phenotype. Results from Chen and colleagues suggest that MANF acts as a negative regulator of inflammation by suppressing NF-κB pathway activation (Chen et al. 2015). They showed that under the condition of inflammation or ER stress MANF was upregulated and translocated into the nucleus where its C-terminal domain interfered with the binding of p65, a subunit of NF-κB, to the target gene promoters. Consequently, MANF suppressed the expression of NF-κB-dependent cytokines and inflammatory cell proliferation. In an *in vitro* model of ischemia, pretreatment of rat primary astrocytes with MANF alleviated oxygen-glucose deprivation induced cell damage, suppressed ER stress and decreased the secretion of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α (Zhao et al. 2013). Anti-inflammatory effects of MANF were also reported in lipopolysaccharide (LPS) -exposed neural stem cells where MANF inhibited inflammatory signaling (IL-1β, TNF-α and IFN-γ) by regulating NF-κB and p38-MAPK pathways (Zhu et al. 2016).

CDNF was shown to be upregulated in rat primary microglia in response to LPS-induced inflammation (Zhao et al. 2014). Cultured microglial cells were exposed to LPS which induced distinct cytotoxicity and secretion of pro-inflammatory cytokines PGE_2 and IL-1β. Pretreatment with CDNF was able to attenuate these reactions, possibly due to suppressed activation of JNK pathway. In agreement with this, overexpression of CDNF in primary astrocytes was sufficient to alleviate ER stress induced cell damage and production of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α (Cheng et al. 2013). The ability of CDNF to limit neuroinflammation has also been observed *in vivo*. In a rat model of spinal cord injury, the transplantation of mesenchymal stem cells overexpressing CDNF was able to decrease the production of PGE_2, IL-1β and TNF-α, promote nerve regeneration and improve the overall motor function of the injured rats (Zhao et al. 2016). Importantly, in a 6-hydroxydopamine (6-OHDA) model of PD in rats, CDNF transfection into the SN reduced glial activation and IL-6 levels in the midbrain (Nadella et al. 2014). Finally, it should be noted that CDNF and MANF may also exert their actions through different mechanisms leading to

synergistic effects as proposed by Cordero-Llana and colleagues based on their observation that combined overexpression of both CDNF and MANF in the SNpc led to more efficacious dopaminergic neuroprotection in 6-OHDA-lesioned rats as compared to the overexpression of either one of the NTFs alone (Cordero-Llana et al. 2015).

2.1.5 Retrograde signaling of neurotrophic factors

In order to regulate neuronal development, plasticity and survival, target-derived NTFs have to be transported retrogradely from the synapses to the cell bodies where they can modulate gene transcription. Because the morphology of neurons is highly polarized, NTF-receptor complexes internalized at the nerve terminals may need to travel long distances along the axons to reach downstream signaling effectors localized in the cell body.

An early study from 1974 showed distal uptake and axonal transport of exogenous radiolabeled NGF and suggested that the retrograde transport of NTFs to neuronal cell bodies plays an important role in their survival promoting effects (Hendry et al. 1974). Later, endogenous unlabeled NGF was also confirmed to be retrogradely transported *in vivo* (Palmatier et al. 1984; Mufson et al. 1999). Upon NGF binding to TrkA receptor, the activated receptor-ligand complex is internalized by clathrin-dependent endocytosis (Ehlers et al. 1995; Grimes et al. 1996; Riccio et al. 1997; Howe et al. 2001; Ye et al. 2003). The endocytic vesicle containing the activated components of MAPK, PLCγ and PI3K/Akt pathways is then retrogradely transported along the axon to the soma by a microtubule-associated protein complex called dynein (Vallee et al. 1989; Bhattacharyya et al. 2002; Vallee et al. 2004). This phenomenon is collectively called “signaling endosome” hypothesis.

Like neurotrophins, GFLs can also provide long-distance trophic support to neurons. For example, in the nigrostriatal pathway GFLs are taken up by axon terminals of dopamine neurons and transported to the cell bodies: receptor-mediated uptake and retrograde transport of GDNF have been demonstrated by injecting biologically active ^{125}I-labeled GDNF into the adult rat striatum and using autoradiography to detect accumulated radioactivity in the ipsilateral SNpc 24h later (Tomac et al. 1995b; Voutilainen et al. 2009, 2011). This suggests that in the adult nigrostriatal system GDNF acts as an endogenous target-derived NTF for dopamine neurons. Similarly, GDNF was shown to be internalized and retrogradely transported by spinal motor neurons in neonatal rats (Yan et al. 1995). In compartmentalized cultures of sympathetic neurons, addition of GDNF to distal axons induced neuronal survival and neurite outgrowth in the cell bodies (Coulpier and Ibáñez 2004). The trophic effects were associated with retrograde transport of GDNF and GFRα1, as well as activation of RET and Akt in the cell bodies suggesting a saturable, receptor-mediated mechanism for the retrograde signaling. A competition study investigating the retrograde transport of ^{125}I-labelled GFLs demonstrated that the transport is mediated selectively by corresponding GFRα co-receptors and only limited cross-binding occurs (Leitner et al. 1999). Proteasomal degradation of RET in distal axons has been suggested to regulate the trophic signaling by modulating the amount of active GFL-GFRα-RET complexes that can be transported to cell bodies (Tsui and Pierchala 2010).

The molecular mechanisms of the retrograde GFL-GFRα-RET signaling are more poorly understood than the mechanism of retrograde NGF-TrkA signaling (Ito and Enomoto 2016). In response to receptor activation, GFL-GFRα-RET complex can be internalized from the plasma membrane via clathrin-mediated endocytosis (Richardson et al. 2006; Richardson et al. 2012; Crupi et al. 2015). The resulting early endosome contains phosphorylated RET and signaling effectors that can activate downstream trophic pathways. It might also anchor to the microtubule network and be transported to the soma by dynein motors in accordance with the "signaling endosome" hypothesis.

Intrastriatally injected CDNF was shown to be internalized by different cortical and striatal neurons, possibly via the endocytic pathway, and undergo retrograde transport to the SNpc along nigrostriatal dopamine neurons (Voutilainen et al. 2011; Mätlik et al. 2017). ^{125}I-labeled CDNF also seemed to spread to the frontal cortex and hippocampus but active transportation to these areas could not be confirmed because the spread was not markedly affected by an excess of unlabeled CDNF (Voutilainen et al. 2011). Intrastriatally injected ^{125}I-MANF, instead, was retrogradely transported, possibly via glutamatergic projection neurons, to the frontal cortex but not to the SN (Voutilainen et al. 2009).

In addition to retrograde transport, there is some evidence of anterograde transport of NTF signaling. In retinal ganglion cells, neurotrophins are anterogradely transported in vesicles along axonal microtubules to the midbrain superior colliculus using conventional kinesin-I motors (Butowt and von Bartheld 2007, 2001). However, the detailed molecular mechanisms and physiological significance of anterograde transport of NTFs remain largely elusive. Interestingly, in many neurodegenerative diseases there are alterations in axonal transport suggesting that defective retro- or anterograde NTF signaling may contribute to neurodegenerative processes (Bronfman et al. 2007; Ito and Enomoto 2016).

2.1.6 Neurotrophic factor mimetics, inducers and variants

Peripherally administered NTFs do not cross the BBB and have short half-lives. An alternative strategy to activate NTF signaling is to use small molecules that bind to and activate NTF receptors or enhance NTF expression. The elucidation of the 3D structures of NTFs and their receptors makes it possible to use rational drug discovery methods in the design and development of such compounds. NTF mimetics potentially afford an opportunity to attain better drug-like properties as compared to recombinant NTFs: Ability to penetrate the BBB would make them suitable for peripheral administration, thus overcoming the high risks associated with the intracranial delivery of NTF proteins or viral vectors. Better diffusion in the brain parenchyma would ensure sufficient dose in the target area, selective binding profile and possibility for intermittent delivery would contribute to a better safety profile. In addition, small molecules would evade the potential risk of immunogenicity related to recombinant protein therapies and would be easier to manufacture in large quantities.

2.1.6.1 GFL mimetics

Considering the potent neurotrophic effects GFLs on midbrain dopamine neurons, targeting GFL-GFRα-RET signaling with GFL mimetics is of therapeutic interest in relation to PD (Lin et al. 1993). The proposed mechanisms of action for a GFL mimetic activating GFRα-RET receptor complex are shown in Figure 2.5. Tokugawa and colleagues described the first small molecule compound, XIB4035, mimicking the biological functions of GDNF (Tokugawa et al. 2003). XIB4035 binds to GFRα1 and activates the GFRα1-RET complex inducing RET phosphorylation and neurite outgrowth in a mouse neuroblastoma cell line. Later, this GDNF mimetic was shown to improve sensory function in animals with advanced neuropathy and to act as a positive modulator of GFRα-mediated signaling instead of being a direct GFRα1 agonist (Hedstrom et al. 2014).

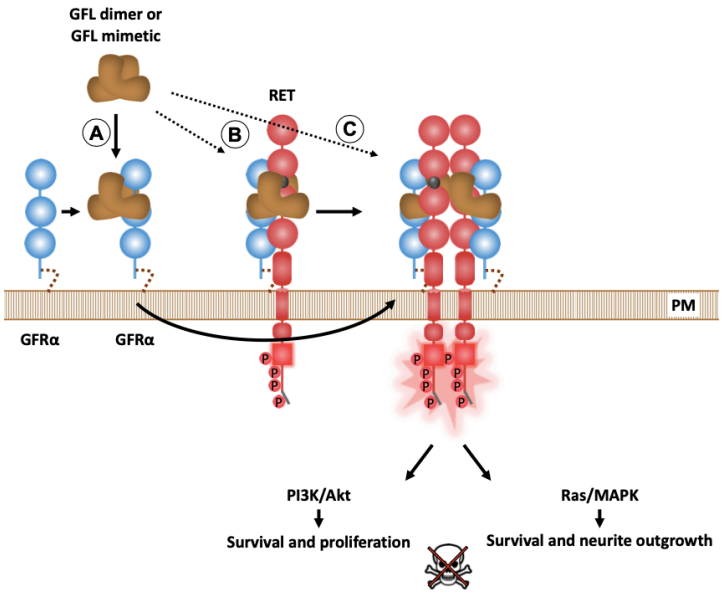

Figure 2.5. Putative mechanisms of action of GDNF family ligand (GFL) mimetics. There are three proposed mechanisms for the activation of GFRα-RET receptor complex by an agonist (i.e. endogenous GFL dimer or GFL mimetic). (**A**) The agonist first binds to one GDNF family receptor α (GFRα) co-receptor, which then engages a second GFRα. Subsequently, the agonist-$(GFR\alpha)_2$ complex recruits two receptor tyrosine kinase RET molecules leading to autophosphorylation of intracellular tyrosine residues of RET, and activation of intracellular signaling pathways like PI3K/Akt and Ras/MAPK. (**B**) The agonist binds to a preformed GFRα-RET heterodimer, and thereby recruit another GFRα-RET pair and activate downstream signaling cascades. (**C**) The receptor complex exists as a preformed $(GFR\alpha)_2$-$(RET)_2$ heterotetramer which acquires a conformational change in response to the binding of the agonist, enabling the phosphorylation of the tyrosine residues of RET. PM - plasma membrane; MAPK - mitogen activated protein kinase; PI3K - phosphatidylinositol 3-kinase. Figure drawn by the author.

A novel family of small molecule GFL mimetics, termed BT13, BT18 and BT44, can selectively activate RET and its downstream signaling pathways (Bespalov et al. 2016; Mahato et al. 2020; Sidorova et al. 2017; Sidorova and Saarma 2020; Viisanen et al. 2020). These compounds have been shown to promote neurite outgrowth from cultured dorsal root ganglia sensory neurons and attenuate hyperalgesia in rat models of neuropathic pain. BT13 also supports the survival of cultured dopamine neurons, protects them from 6-OHDA and 1-methyl-4-phenylpyridinium (MPP+) induced cell death in a RET-dependent manner, and is able to stimulate dopamine release

in the mouse striatum (Mahato et al. 2020). The preferred binding sites of BT13 in the GFRα1-RET complex were investigated using molecular docking simulations and identified to be the allosteric modulation site of GFRα1 and the GFRα1-interacting site of RET (Ivanova et al. 2018). The latter, more likely binding site, enables BT13 to act as a direct RET agonist.

2.1.6.2 NTF inducers

A large number of compounds, both pharmacological and natural agents, has been suggested to stimulate endogenous NTF synthesis, which potentially has disease-modifying relevance in PD management. These compounds are extensively reviewed by Saavedra et al. (2008) and include, but are not limited to, dopamine agonists, monoamine oxidase (MAO) inhibitors, catecholamine reuptake inhibitors, anti-depressants, anti-psychotics, vitamin D_3, estrogen and various medicinal herb extracts. Rasagiline, a clinically used MAO-B inhibitor, is one of such compounds that has attracted attention. It seems to upregulate BDNF and GDNF levels *in vitro* and *in vivo* (Bar-Am et al. 2005; Maruyama et al. 2004; Weinreb et al. 2009). In a rat 6-OHDA model of PD, rasagiline protected nigrostriatal dopamine neurons and alleviated motor decline (Blandini et al. 2004). Likewise, in a mouse lactacystin model of PD, rasagiline showed neuroprotective and neurorestorative effects and induced motor recovery (Zhu et al. 2008). Rasagiline has also been tested in early-stage PD patients in two phase II trials with delayed-start design (Parkinson Study Group 2004; Olanow et al. 2009; Hauser et al. 2009). It showed some disease-modifying effects, but further evidence is needed for decisive conclusions.

Dopaminergic neurotransmission itself is proposed to regulate the expression of GDNF and BDNF in the nigrostriatal system (Saavedra et al. 2008). For example, D1 and D2 receptor agonists increase the synthesis of GDNF in midbrain and striatal primary cultures (McNaught and Jenner 2000; Ohta et al. 2000; Guo et al. 2002; Ohta et al. 2004). Consequently, striatal dopamine depletion may lead to downregulation of GDNF which can contribute to the disease progression in PD patients. Clinically used D2-like dopamine receptor agonists pramipexole and ropinirole have been speculated to have neurotrophic properties. In rodent models of PD, they protected nigrostriatal dopamine neurons against 6-OHDA- and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) -induced toxicity (Iida et al. 1999; Zou et al. 2000; Anderson et al. 2001; Ramirez et al. 2003). It should be noted, however, that the neuronal uptake of 6-OHDA and MPTP via DAT is a prerequisite for their toxic effects and that the neuroprotection seen in these models could be attributed to the regulation of DAT function by presynaptic D2 autoreceptor activation (Joyce et al. 2004). Neuroimaging evidence from clinical studies also suggests that pramipexole and ropinirole can affect the disease progression (Clarke and Guttman 2002; Parkinson Study Group 2002; Whone et al. 2003). The clinical relevance of these observations, however, has remained insignificant.

2.1.6.3 NTF variants

Structurally modified or truncated NTF variants have been engineered in search of improved pharmacokinetic properties of NTFs (Piltonen et al. 2009; Runeberg-Roos et al. 2016). Insufficient distribution in the brain parenchyma has been proposed as one major factor contributing to the

lack of efficacy in the phase II clinical trials with GDNF and NRTN. As mentioned earlier, the diffusion of GDNF and NRTN in the brain tissue is hindered by their high affinity binding to heparan sulfates in the extracellular matrix. Thus, the promising neuroprotective effects of these GFLs in relatively small rat brain may not necessarily translate to much larger human brain where the GFLs have to diffuse long distances from the site of application.

To improve the diffusion properties of GFLs, biologically active variants with reduced heparin binding affinity have been investigated (Piltonen et al. 2009; Smith et al. 2015; Runeberg-Roos et al. 2016; Grondin et al. 2019). N-terminally truncated, non-heparin-binding Δ38N-GDNF was more widely distributed in the rat striatum than wild-type GDNF, stable in a brain extract stability assay and protective against amphetamine-induced turning behavior in a 6-OHDA model of PD (Piltonen et al. 2009). Nevertheless, the Δ38N-GDNF was unable to significantly promote the survival of dopaminergic cell bodies in the SNpc. Overall, widespread striatal diffusion of Δ38N-GDNF did not improve its efficacy as compared to wild-type GDNF. These data suggest that although heparan sulfate binding of GDNF restricts its distribution in the brain, it may be needed for the optimal neuroprotective effects.

Another GDNF variant (GDNFv) was engineered from wild-type GDNF by removing 31 amino acids from the N-terminal domain to reduce heparin binding and substituting two conservative amino acids (N38Q and D95E) to improve chemical stability (Smith et al. 2015). These modifications resulted in significantly improved distribution in the rat brain as well as in the larger rhesus macaque brain after intrastriatal administration, better chemical stability and lower predicted immunogenicity as compared to wild-type GDNF. *In vitro*, GDNFv was equivalent to wild-type GDNF in GFRα1 receptor binding, RET phosphorylation and neurite outgrowth assays. In a 6-OHDA model of PD in rats, its ability to protect dopamine neurons was comparable to wild-type GDNF, and in naïve rhesus macaques GDNFv enhanced dopamine turnover (Grondin et al. 2019).

Structural modifications of NRTN were shown to increase its diffusion properties in the rat and monkey brain as compared to wild-type NRTN (Runeberg-Roos et al. 2016). This NRTN variant was also able to restore dopamine fibers in the striatum and alleviate motor impairment in 6-OHDA-lesioned rats more potently than GDNF showing promise for further development of NTF variants into disease-modifying therapies for PD.

2.2 Basal ganglia circuitry and dopaminergic system

Basal ganglia are comprised of a highly conserved chain of subcortical nuclei in the forebrain that orchestrate action selection, motor coordination, habit formation, learning and motivation (Gerfen and Surmeier 2011; Macpherson and Hikida 2019). They are in a central position to facilitate voluntary movements and to inhibit interfering or competing movements. Hence, the functions mediated by the basal ganglia cover behaviors that are vital for animal survival. The largest nucleus of the basal ganglia is the striatum. Based on the distinct functions and connections, the striatum is subdivided into the dorsal striatum consisting of the caudate nucleus and putamen, and the ventral striatum including the nucleus accumbens (NAcc) and olfactory tubercle. The other basal ganglia nuclei include the pallidal nuclei, that are comprised of the

external and internal segment of the globus pallidus (GPe and GPi, respectively) and the ventral pallidum, subthalamic nucleus (STN), substantia nigra pars compacta and pars reticulata (SNpc and SNr, respectively) and ventral tegmental area (VTA). Perturbations in basal ganglia functions underlie debilitating movement disorders that are characterized by impaired voluntary movements, the presence of involuntary movements or both, such as in PD and Huntington's disease (Macpherson and Hikida 2019). Dysfunctions of the basal ganglia also give rise to a spectrum of psychiatric conditions including schizophrenia, depression, anxiety, obsessive-compulsive disorder and addiction.

2.2.1 Organization of the basal ganglia circuits

Basal ganglia circuitry receives its information from the cortex, modulates it under the control of midbrain dopaminergic innervation, and sends it back to the cortex through the thalamus (Gerfen and Surmeier 2011; Macpherson and Hikida 2019). The primary input nucleus of the basal ganglia is the striatum. It is anatomically connected to the cerebral cortex, thalamus and limbic system, and receives direct glutamatergic inputs from all main cortical areas (motor, sensory and associational areas) as well as thalamic nuclei. Midbrain dopamine neurons located in the SNpc and VTA send wide and dense dopaminergic innervation to the striatum where it merges with and modulates the cortical and thalamic glutamatergic input (Hooks et al. 2018; Klaus et al. 2019). The axonal arborization of a single dopamine neuron is shown to cover up to 6% of total striatal volume in rats (Matsuda et al. 2009). The basic organization of the basal ganglia circuitry is illustrated in Figure 2.6.

The striatum hosts two groups of GABAergic medium spiny neurons (MSNs; also called spiny projection neurons, SPNs) which give rise to two parallel projection pathways that convey information from the striatum to the output nuclei of the basal ganglia, i.e. the GPi and SNr (Gerfen and Surmeier 2011; Macpherson and Hikida 2019). The GPi and SNr are the final information-processing nuclei before output signals are sent to the ventral lateral nucleus and ventral anterior nucleus of the thalamus, superior colliculus and pedunculopontine nucleus (PPN) via GABAergic projections. The GPi and SNr are often considered as one somatotopic structure (the GPi/SNr), where the GPi is responsible for axial and limb movements and the SNr for head and eye movements.

The excitatory corticostriatal glutamatergic input arises from the cortex layer 5 pyramidal neurons and is directed to the dendritic spines of the MSNs. Similarly, thalamostriatal glutamatergic projections arise from the intralaminar thalamic nuclei and form synapses on the dendritic shafts and spine heads of the MSNs. These inputs are modulated by dopaminergic input from the nigrostriatal pathway which is directed to the spine necks of MSNs (Figure 2.8). Approximately half of the striatal MSNs express excitatory dopamine D1 receptors, substance P and dynorphin (D1-MSNs), and form the "direct" striatonigral pathway that projects monosynaptically to the GPi/SNr (Gerfen and Surmeier 2011; Macpherson and Hikida 2019). The other half expresses inhibitory dopamine D2 receptors and enkephalin (D2-MSNs) and project polysynaptically to the GPi/SNr forming the "indirect" striatopallidal pathway (Figure 2.6). D2-MSNs first extend axonal

projections to the GABAergic neurons of the GPe, which in turn provide inhibitory innervation to the STN. Thereafter, glutamatergic neurons of the STN send excitatory projections to the GPi/SNr.

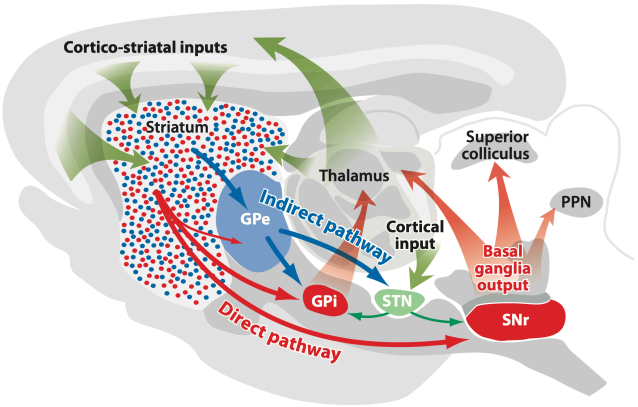

Figure 2.6. Simplified diagram of the basal ganglia-thalamocortical circuitry. The striatum functions as an integrator of the basal ganglia input signals. It receives excitatory glutamatergic input from the cortex and thalamus (wide green arrows). In addition, dopaminergic input from the substantia nigra pars compacta (SNpc) arrives to the striatum (not shown) and modulates the glutamatergic input. The inhibitory GABAergic outputs of the basal ganglia project from the internal segment of the globus pallidus (GPi) and substantia nigra pars reticulata (SNr) to thalamus, superior colliculus and pedunculopontine nucleus (PPN) (wide red arrows). The direct pathway arises from GABAergic D1 receptor expressing medium spiny neurons (MSNs) and projects directly to the output nuclei GPi and SNr (thin red arrows). The indirect pathway originates from GABAergic D2 receptor expressing MSNs that project only to the external segment of the globus pallidus (GPe) (thin blue arrows). The GPe further sends GABAergic afferents to the subthalamic nucleus (STN), which connects the signal to the output nuclei via glutamatergic projections (thin green arrows). Also, hyperdirect pathway providing glutamatergic input from the cortex to the STN (wide green arrow), direct pathway axon collaterals from the striatum to the GPe (thin red arrow), and collaterals from the GPe to the output nuclei (thin blue arrow) are shown in the diagram. Reproduced with the permission of Annual Reviews, from Gerfen and Surmeier (2011), Annual Review of Neuroscience, © Annual Reviews 2011.

Evidence from optogenetical and electrophysiological measurements has shown, that direct pathway D1-MSNs also extend axon collaterals from the dorsal striatum and NAcc to the GPe and ventral pallidum, respectively, indicating that the canonical “direct/indirect” architecture of the striatal output pathways is more complex than originally though (Gerfen and Surmeier 2011; Kupchik et al. 2015). Moreover, the GPe sends some GABAergic projections directly to the GPi/SNr, as well as back to the striatum, where they are able to inhibit striatal movement representations (see below chapter 2.2.4) (Bevan et al. 1998; Gerfen and Surmeier 2011; Mallet et al. 2016, 2012). The STN also receives glutamatergic input in addition to GABAergic input from the GPe. These glutamatergic projections originate from the cortex forming the “hyperdirect” pathway that bypasses the striatum (Nambu et al. 2002). The hyperdirect pathway has been proposed to be critical for suppressing thalamocortical output, and thereby inhibiting erroneous or competing motor programs (Nambu et al. 2002; Aron and Poldrack 2006). Dopamine neurons of the ventral tier of the SNpc also send dendrites through the SNr where they are able to release dopamine (Zhou et al. 2009). This dopaminergic connection between the SNpc and SNr is termed the “ultra-short pathway”. The dendritic dopamine release can activate presynaptic D1 receptors on the striatonigral MSN terminals which enhances GABA release in the SNr, and thus further suppresses the activity of nigral output projections to the thalamus (Robertson 1992).

2.2.2 Functions of the basal ganglia circuits

The dorsal striatum is mainly responsible for cognition involving motor and executive functions, while the ventral striatum (especially the NAcc) primarily controls motivation, reward, positive reinforcement and Pavlovian-instrumental conditioning (Macpherson and Hikida 2019). The dorsal striatum is traditionally further divided into dorsomedial region (DMS) and dorsolateral region (DLS) based on anatomical and functional characteristics (McGeorge and Faull 1989). DMS receives input from the frontal- and parietal-associative cortices and plays an important role in skill acquisition and goal-oriented behavior (Gerfen and Surmeier 2011). DLS, on the other hand, receives the main input from the sensorimotor cortex, and this loop is responsible for automated action programs, habit formation and locomotion. Additionally, the basolateral amygdala sends excitatory afferents to the dorsal striatum which, according to experiments in non-human primates (NHPs), seem to modulate striatal activity based on the emotional context and internally generated goals (Kelley et al. 1982; Pan et al. 2010; Hernádi et al. 2015; Maeda et al. 2018). The NAcc receives projections from the limbic structures such as prefrontal and anterior cingulate cortices, amygdala and hippocampus. Recent developments in chemogenetic and optogenetic tools, viral tracers and electrophysiological techniques have enabled further division of the striatum into smaller functional subregions beyond the simplified ventral-dorsal and medial-lateral divisions (Chuhma et al. 2019).

Besides the anatomical and functional organization, the dorsal and ventral striatum can be neurochemically divided into two main compartments known as striosomes and matrix (Brimblecombe and Cragg 2017; Crittenden and Graybiel 2016; Graybiel and Ragsdale 1978). Striosomes occupy on average 10−15% of the striatal volume and form small labyrinth-like zones that are surrounded by larger matrix compartment. Striosomes are defined histochemically based on high expression of e.g. μ-opioid receptors, substance P, D1 receptors and calretinin. Matrix, by contrast, is enriched with acetylcholinesterase, choline acetyltransferase, calbindin, somatostatin and D2 receptors. Both compartments contain direct and indirect pathway MSNs. The most prominent difference in the neuronal connectivity of the two compartments is the direct D1-MSN projection from striosomes to dopamine neurons of the ventral tier of the SNpc forming striato-nigro-striatal loop. Striosomes also receive more innervation from the ventral tier, and matrix from the dorsal tier of the SNpc. Interestigly, studies in rats suggest that dopamine levels and TH-immunoreactivity are higher in matrix but the density of dopaminergic axons is greater in striosomes. In addition to the dopaminergic innervation, striosomes receive abundant input from the limbic areas of the prefrontal cortex, whereas matrix receives cortical inputs primarily from the sensorimotor and associative cortices. The inputs from the thalamus and amygdala are also dissimilar between the two compartments. Studies of functional differences between striosomes and matrix are still at an early stage. Striosome-related circuits are proposed to regulate motivation, behavioral flexibility and choice under stressed conditions (Crittenden and Graybiel 2016). The two compartments are also suggested to be differentially involved in pathological processes in a range of psychomotor disturbances including PD, Huntington's disease, attention deficit and hyperactivity disorder (ADHD).

As described above, the striatum receives dense and convergent excitatory inputs from multiple cortical areas and thalamic nuclei. Thus, it serves as an integrator of this sensorimotor, cognitive and motivational information, selects the most appropriate behaviors and translates them into specific changes in the basal ganglia outputs. Cortico-basal ganglia neurocircuits can be divided into three functional loops, the sensorimotor, associative/cognitive and limbic loops, which play different roles in context-dependent action planning, selection and initiation (Gerfen and Surmeier 2011; Macpherson and Hikida 2019).

According to the classical model of action selection the basal ganglia tonically inhibit downstream motor centers (i.e. the motor cortex, superior colliculus and PPN) via GABAergic output projections, thus preventing the occurrence of unwanted or wrongly timed movements (Albin et al. 1989; Gerfen and Surmeier 2011; Klaus et al. 2019). Cortical and thalamic inputs give rise to the activation of the direct and indirect pathways which exert opposing effects on output nuclei. The activation of the direct pathway causes pauses in the tonic inhibition of the output projections enabling the desired movements, whereas the indirect pathway further suppresses the competing actions. Nigrostriatal dopamine release dichotomously modulates the direct and indirect projections facilitating the movement initiation.

Traditionally, rapid phasic activity of the midbrain dopamine neurons is suggested to drive reward, learning and transitions between movement states (Jin and Costa 2010; Matsumoto and Hikosaka 2009; Schultz 2007). Brief increase in the firing rate of the SNpc neurons and subsequent transient fluctuation in the striatal dopamine level occur immediately before or around self-paced movement initiation (Howe and Dombeck 2016; da Silva et al. 2018). It was recently shown that the transient increase in dopaminergic activity immediately preceding movement initiation correlates with the vigor of the initiated movement (da Silva et al. 2018). Brief optogenetic activation of the SNpc dopamine neurons before movement initiation, but not during an ongoing movement, increased the probability and vigor of future movements, while optogenetic inhibition resulted in less probable and less vigorous movements.

The classical model is challenged by accumulating evidence suggesting that both direct and indirect projection pathways are coactivated during movement and inhibited during immobility (Klaus et al. 2019). The new model proposes that both direct and indirect pathways might work in concert to facilitate the desired motor actions and suppress the unwanted movements by concurrently activating different basal ganglia output neurons. The cortex represents alternative motor plans to specific striatal MSN ensembles. Action specificity arises from the pattern of cortical inputs, while dopaminergic inputs nonspecifically modulate the excitability of the MSNs. Only with adequate phasic dopamine activity specific MSN ensembles become active depending on the strength of their cortical inputs. Too low dopaminergic input results in subthreshold striatal output despite the presence of strong cortical signals. In this model, the relative timing and activity levels of specific MSN ensembles are thought to have a critical impact on the final basal ganglia output signal. However, new cell type -specific recordings with single-cell resolution have demonstrated that the spatiotemporal dynamics of MSN subtypes during natural behavior is action-specific and rather complex (Klaus et al. 2017; Markowitz et al. 2018; Parker et al. 2018).

The abovementioned models of the basal ganglia functions are based on the current understanding of the circuitry and provide an oversimplified picture of their true physiology. The descriptions provided here, for example, completely ignore the fact that the GPe, GPi, STN and SNr neurons can also serve as autonomous pacemakers generating action potentials without synaptic input, consequently adding another dimension to the modulation of the basal ganglia circuitry (Gerfen and Surmeier 2011). There are still a great many questions to be answered before we can fully understand the role of the basal ganglia in action selection and motor control.

2.2.3 Striatal interneurons

The distribution of the neuron populations is fairly uniform throughout the striatum: MSNs comprise 90-95% of the striatal neurons, and the remaining 5-10% are interneurons (Gerfen and Surmeier 2011; Chuhma et al. 2019). Despite the relatively small population, striatal interneurons have an important role in regulating the striatal output. Their behavioral relevance, however, remains poorly understood thus far.

Striatal interneurons can be divided into large cholinergic interneurons and three types of smaller GABAergic interneurons: (a) fast spiking (FS) PV-positive, (b) low-threshold spiking (LTS) somatostatin-, neuropeptide Y- and nitric oxide synthase-positive, and (c) calretinin-positive interneurons (Kawaguchi et al. 1995; Tepper et al. 2008; Kreitzer 2009). FS interneurons are most abundant in the rat striatum and their electrophysiological properties are dominated by short-duration action potentials, abrupt high-frequency firing and hyperpolarized resting potential. FS interneurons receive excitatory synapses from the cortex and thalamus and inhibitory inputs from other interneurons and a subpopulation of GPe neurons. In addition, dopamine and acetylcholine can increase their activity via D5 and nicotinic acetylcholine receptors (nAChRs), respectively. Gap junctions between the dendrites contribute to electrotonic coupling that can lead to synchronous firing among local FS interneuron populations.

LTS interneurons are physiologically distinguished from the FS cells by less negative resting potential, long-duration action potentials and higher input resistance (Kawaguchi et al. 1995; Kreitzer 2009). Like FS interneurons, LTS interneurons are also innervated by glutamatergic afferents from the cortex and thalamus and form synapses with MSNs. Dopamine can modulate the activation of LTS neurons via D5 receptors, and acetylcholine can exert either activating or inhibiting modulation via muscarinic M1 or M2 receptors, respectively. Due to the wide variety of transmitters, LTS interneurons can affect the activity of MSNs not only through the release of GABA but also nitric oxide, somatostatin and neuropeptide Y.

Cholinergic interneurons constitute only approximately 1–2% of striatal cells, but their effects are much more significant (Kreitzer 2009). They show tonic spontaneous firing on average at 2-10 Hz, long-lasting afterhyperpolarizations, depolarized resting potential and relatively high input resistance. Cholinergic interneurons receive excitatory innervation primarily from the intralaminar thalamic nuclei and, to a lesser extent, from the cortex as well as inhibitory synapses from MSNs. Their widespread axonal arborization connects mainly with D1- and D2-MSNs, but also with FS interneurons, through perisomatic synapses forming the feedforward thalamostriatal circuit

(Gerfen and Surmeier 2011). Nigrostriatal dopamine release modulates the activity of cholinergic interneurons through D2 and D5 receptors. D2, together with M2 and M4 receptor signaling, reduces their excitability (Calabresi et al. 1998; Chuhma et al. 2014; Maurice et al. 2004), whereas D5 receptor activation stimulates them (Aosaki et al. 1998). M2 also functions as a presynaptic autoreceptor regulating acetylcholine release (Hersch et al. 1994). Cholinergic interneurons can modulate direct-pathway MSNs through their M1 and M4 receptors and indirect-pathway MSNs through M1 receptors (Bernard et al. 1992; Yan and Surmeier 1996), as well as dopaminergic transmission through nAChRs on dopaminergic axon terminals (Wonnacott et al. 2000; Jones et al. 2001; Zhou et al. 2001; Cachope et al. 2012; Threlfell et al. 2012; Mamaligas et al. 2016). Thus, dopamine and acetylcholine together exert complex modulation of neuronal activity in the striatum that strongly influences basal ganglia output. On top of that, other neuromodulators such as adenosine, endocannabinoids, nitric oxide and various neuropeptides play important roles in shaping the final output signals.

2.2.4 Striatal microcircuits regulating the direct and indirect pathways

There are three distinct inhibitory microcircuits in the striatum that counterbalance the glutamatergic excitation of the MSNs (Gerfen and Surmeier 2011). They are thought to contribute to action selection by suppressing unwanted activities. Firstly, the feedforward inhibitory circuit originates from corticostriatal inputs that enhance the activity of FS interneurons (Mallet et al. 2005). The interneurons then form perisomatic GABAergic synapses on both D1- and D2-MSNs and effectively reduce their spiking. Secondly, FS interneurons receive GABAergic innervation from the GPe neurons forming the inhibitory pallidostriatal feedback circuit (Gerfen and Surmeier 2011). On this feedback circuit, dopamine exerts dual excitatory actions by directly depolarizing the interneurons through D5 receptors and reducing their synaptic inhibition through presynaptic D2 receptors on pallidostriatal nerve terminals (Bracci et al. 2002; Centonze et al. 2002, 2003; Wiltschko et al. 2010). Thirdly, the striatal feedback circuit involves local axon collaterals of D1- and D2-MSNs that make synaptic connections with the dendrites of other MSNs providing lateral inhibition (Gerfen and Surmeier 2011). The release of GABA on these collateral synapses is regulated by dopamine; D2 receptor activation decreases and D1 activation increases the release of GABA. Taken together, we can appreciate the complex role of striatal interneurons in regulating the activity of direct- and indirect-pathway MSNs. Their exact effects on the basal ganglia circuitry are yet to be resolved.

2.2.5 Dopamine cell groups and projection pathways

Arvid Carlsson, Eric Kandel and Paul Greengard were awarded the Nobel Prize in medicine and physiology in 2000 for their seminal work in the field of catecholaminergic neurotransmission in the late 1950s that lead to recognition of dopamine as an independent neurotransmitter in the brain (Carlsson 2001, 1959; Carlsson et al. 1958; Sano et al. 1959). Soon after that, in 1964, Dahlström and Fuxe mapped for the first time the locations of monoamine-containing cell bodies and nerve terminals in the rat brain (Dahlström and Fuxe 1964). Later, the development of sensitive immunohistochemical and tract-tracing techniques has provided a more detailed picture of the dopamine system making it one of the most completely mapped neurotransmitter systems

in the brain. The current understanding of the nine different dopamine-containing neuronal groups (groups A8–A16) in the mammalian brain is reviewed in (Björklund and Dunnett 2007) and depicted in Figure 2.7.

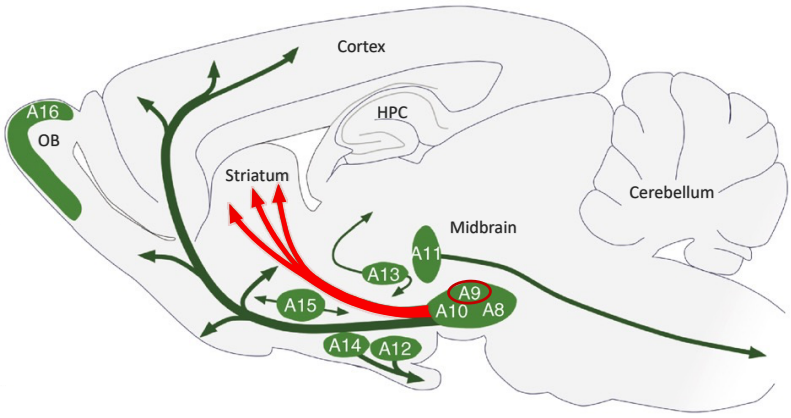

Figure 2.7. Dopamine neuronal groups and dopaminergic pathways in the rat brain. Dopamine neuron cell bodies are localized in nine distinctive groups (A8-A16), distributed from the midbrain to olfactory bulb (OB). The nigrostriatal pathway connecting dopamine neurons located in the substantia nigra pars compacta (A9) to the dorsal striatum is highlighted with wide red arrows and a circle. The mesolimbic and mesocortical pathways from the ventral midbrain dopamine neuron groups are illustrated by wide green arrows. The tuberoinfundibular pathway from the A12 and A14 groups and the diencephalospinal pathway from the A11 group are shown by thin green arrows. The cell groups are defined based on tyrosine hydroxylase immunoreactivity. HPC – Hippocampus. Adapted with permission from Elsevier: TRENDS in Neurosciences, Björklund and Dunnett (2007), © Elsevier 2007.

Ventral midbrain dopamine neurons are arranged in three different groups: the retrorubral field (A8 group), the SNpc (A9 group) and the VTA (A10 group) (Björklund and Dunnett 2007). These neuron populations project to defined areas of the forebrain and modulate specific functions according to their target areas. The dopaminergic projections are traditionally divided into three major pathways: Dopamine neurons located in the SNpc mainly innervate the dorsal striatum, forming the nigrostriatal pathway (Fallon and Moore 1978; Björklund and Dunnett 2007; Howe and Dombeck 2016). The nigrostriatal pathway is predominantly responsible for the regulation of motor functions and is implicated in the pathophysiology of PD (see chapter 2.3.2). Dopamine neurons located in the VTA primarily project to the ventral striatum (NAcc and olfactory tubercle) and cortical areas (prefrontal cortex and cingulate cortex) forming the mesolimbic and mesocortical pathways, respectively. These pathways regulate emotional behavior, motivation, reward, learning and cognitive functions, and are afflicted in an array of psychiatric disorders (Hornykiewicz 1978; Parker et al. 2016; Macpherson and Hikida 2019). The retrorubral field is a dorso-caudal extension of the SNpc and projects to both striatal, limbic and cortical areas (Björklund and Dunnett 2007).

The reality of the anatomical organization and projection patterns of the dopamine neurons in the midbrain cell groups is more complex than the original division described above (Loughlin and Fallon 1984; Swanson 1982). The cell groups can be further divided into different subcompartments (such as the dorsal and ventral tier of the SNpc and VTA) that differ in their cellular morphology, some molecular markers and projection patterns. For example, the dorsal

tier of the SNpc also contains neurons that send afferents to limbic and cortical areas (François et al. 1999). Using sophisticated tissue clarification, optogenetic and fiber photometry tools Lerner and colleagues identified two parallel dopamine neuron subpopulations within the SNpc with divergent biophysical properties and distinct output projections to the DMS and DLS (Lerner et al. 2015). The intermixing of the cell bodies of the nigrostriatal, mesolimbic and mesocortical pathways is more prominent in NHPs than in rodents (Williams and Goldman-Rakic 1998; François et al. 1999). Midbrain dopamine neurons also send sparse collateral innervation to several other basal ganglia structures including the GPe, GPi and STN enabling dopaminergic modulation of the basal ganglia circuitry at pallidal and subthalamic levels (Lindvall and Björklund 1979; Lavoie et al. 1989; Smith et al. 1989; Hassani et al. 1997; Gauthier et al. 1999; Prensa and Parent 2001).

The total number of TH-ir cells in the ventral midbrain (in A8, A9 and A10 groups together, bilaterally) is approximately 20 000–30 000 in mice and 40 000–45 000 in rats (Björklund and Dunnett 2007). About half of those cells are located in the SNpc. In humans, the same numbers are approximately 400 000–600 000, with >70% of the neurons located in the SNpc. The larger number of the midbrain dopamine neurons in humans is accompanied, for example, with much more extensive mesocortical innervation as compared to rodents.

Dopamine neurons in the diencephalon are divided into four groups: A14 (the rostral hypothalamic periventricular group), A13 (the dorsal hypothalamic group of the medial zona incerta), A12 (the infundibular group of the arcuate nucleus), and A11 (the posterior and dorsal hypothalamic group and the periventricular grey matter of the caudal thalamus) (Björklund and Nobin 1973). The best known projection pathway arising from these cell groups is the tuberoinfundibular pathway which originates from the A12 and A14 dopamine neurons and innervates the median eminence and the neural lobe of the pituitary gland (Goldsmith et al. 1990; Grattan 2015). Dopamine released from the tuberoinfundibular projections suppresses the secretion of prolactin from anterior pituitary gland by binding to D2 receptors.

The A11 group gives rise to diencephalospinal dopamine system. The A11 neurons project to all levels of the spinal cord providing the only source of inhibitory dopaminergic innervation for the spinal cord (Barraud et al. 2010; Puopolo 2019; Qu et al. 2006; Skagerberg and Lindvall 1985). Descending fibers innervate both the dorsal and ventral horn of the spinal cord and have an important role in sensorimotor integration and pain control. Dysfunction of the A11 dopamine system is suggested to have implications in the pathophysiology of pain (Fleetwood-Walker et al. 1988; Puopolo 2019), migraine (Charbit et al. 2009; Charbit et al. 2010) and restless legs syndrome (Clemens et al. 2006; Lanza et al. 2017).

The functions of the A13 group dopamine neurons and their so called incertohypothalamic projections are less well established (Fougère et al. 2019). They have been suggested to provide local hypothalamic innervation and send projections to locomotor centers of the brainstem. They may be associated with visceral and sensorimotor activities. The A15 group in the rostral hypothalamus contain TH-ir neurons but not detectable levels of dopamine or noradrenaline (Björklund and Dunnett 2007). The A15 neurons give rise to local diencephalic innervation but

their functional role has remained rather obscure. There is some evidence of their role in hormonal regulation (Clarkson and Herbison 2011; Liu and Herbison 2013; Brown et al. 2015).

Finally, the A16 group located in the olfactory bulb hosts the most numerous population of dopamine neurons in the brain (Pignatelli and Belluzzi 2017). Dopamine neurons form the most external (glomerular) layer of the olfactory bulb and function as interneurons co-releasing dopamine and GABA from separate vesicle pools. They modulate the activity of the olfactory nerve terminals and play a key role in odor processing. Interestingly, the A16 cells are regenerated throughout the lifetime; the RMS originating from the SVZ provides a constant source of new dopamine neurons to be integrated to the neuronal network of the olfactory bulb.

2.2.6 Dopamine receptors and modulatory effects

2.2.6.1 Five dopamine receptors

Reflecting the dense dopaminergic innervation from the ventral midbrain, the striatum has a prominent expression of dopamine receptors. The dopamine receptors are metabotropic G protein-coupled receptors and can be divided into two classes on the basis of the G-protein to which they couple: D1-like receptors (D1 and D5) are stimulatory and couple to G_s and G_{olf} proteins, whereas D2-like receptors (D2, D3 and D4) are inhibitory and couple to G_i and G_o proteins (Neve et al. 2004). G_s and G_{olf} proteins stimulate adenylate cyclase enzyme which elevates intracellular levels of cyclic adenosine monophosphate (cAMP). cAMP further activates protein kinase A (PKA) which has a broad range of cellular targets, including receptors, enzymes, ion channels and transcription factors. D1 receptor may also activate phospholipase C (PLC) in a cAMP-independent manner by interacting with G_q. PLC catalyzes the hydrolysis of a membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to two secondary messengers, diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP3).

G_i and G_o target many effectors such as ion channels, phospholipases and receptor tyrosine kinases through a membrane-delimited mechanism involving the liberation of $G_{\beta\gamma}$ subunits (Neve et al. 2004). For example, D2 receptor stimulation in striatal MSNs activates PLC via $G_{\beta\gamma}$ subunits leading to mobilization of intracellular Ca^{2+} storages. As a result, transmembrane Ca^{2+} currents through L-type Ca^{2+}-channels are suppressed and neuronal excitability reduced (Hernández-López et al. 2000). $G_{i/o}$ is also negatively coupled to adenylate cyclase leading to decreased levels of cAMP and activate protein phosphatases that directly counter the effects of PKA (Neve et al. 2004). Thus, the activation of D1-like and D2-like receptors leads to opposing cellular responses.

All five dopamine receptors (D1-D5) are expressed in the striatum, but D1 and D2 are by far the most abundant (Gerfen and Surmeier 2011). Their expression in direct- and indirect-pathway MSNs is dichotomous: direct-pathway MSNs express excitatory D1 receptors and indirect-pathway MSNs inhibitory D2 receptors. D1 and D2 receptors have different binding affinities (Macpherson and Hikida 2019). The high-affinity D2 receptors are occupied at lower dopamine concentrations (~10 nM) and respond more readily to tonic basal dopamine levels (~10-30 nM) inhibiting D2-MSNs. The low-affinity D1 receptors, instead, are primarily activated by increased phasic

dopamine release (at ~1 μM) transiently enhancing the responsiveness of D1-MSNs (Surmeier et al. 2007). Hence, phasic dopamine release has an opposite effect on the activity of MSNs. D2 receptor signaling decreases the responsiveness of indirect-pathway MSNs to uncoordinated cortical activity, whereas transient D1 receptor signaling increases the excitability of the direct-pathway MSNs contributing to properly coordinated action initiation.

D2 receptors expressed at presynaptic axon terminals function as autoreceptors regulating synaptic neurotransmission. D2 autoreceptor activation inhibits the opening of voltage-gated Ca^{2+} channels resulting in decreased axonal dopamine release (Cardozo and Bean 1995). Additionally, D2 autoreceptor activation decreases dopamine release by other mechanisms that include inhibiting dopamine synthesis, enhancing dopamine reuptake via DAT and regulating vesicular monoamine transporter (VMAT) expression (Schmitz et al. 2003).

2.2.6.2 Dopaminergic modulation of synaptic plasticity

In addition to modulating the short-term activity of the corticostriatal circuits, dopamine has an important part to play in regulating the long-term weakening and strengthening of synaptic transmission, i.e. LTD and LTP, respectively (Gerfen and Surmeier 2011). These synaptic plasticity events are believed to be the underlying mechanisms leading to learning and habit formation. Transient elevation in striatal dopamine release decreases the excitability of D2-MSNs and promotes the induction of LTD at corticostriatal synapses. LTD is also regulated by cholinergic interneurons and postsynaptic release of endocannabinoids which activate presynaptic CB1 cannabinoid receptors causing a decrease in glutamate release (Gerdeman et al. 2002; Tozzi et al. 2011).

In contrast, at the glutamatergic synapses of D1-MSNs, activation of D1 receptors increases the excitability and promotes the induction of LTP (Gerfen and Surmeier 2011). LTP seems to depend on the coactivation of D1 and NMDA receptors, as well as BDNF-TrkB signaling (Kerr and Wickens 2001; Calabresi et al. 2007; Jia et al. 2010). D1 receptor activation reinforces the function of AMPA and NMDA receptors and enhances their surface trafficking which is likely to contribute to the induction of LTP (Snyder et al. 2000; Sun et al. 2005; Hallett et al. 2006). Hence, the plastic changes caused by transient elevation in striatal dopamine release promotes the ability of cortical input to activate direct-pathway MSNs (LTP) and reduces its ability to turn on indirect-pathway MSNs (LTD). Ultimately, both changes facilitate movement initiation.

2.2.7 Dopamine lifecycle

2.2.7.1 Dopamine biosynthesis and its regulation

Although dopamine is an important neurotransmitter in the brain, almost half of dopamine in the body is synthesized in the gastrointestinal tract (Eisenhofer et al. 1997). Here, the focus is on dopamine lifecycle in the CNS which is summarized in Figure 2.8. The biosynthesis of dopamine takes place in the cytosol of catecholaminergic neurons in a two-step reaction (Meiser et al. 2013). In the first step, TH catalyzes the hydroxylation of L-tyrosine at the phenol ring to L-3,4-

dihydroxyphenylalanine (L-DOPA). For this, TH requires a cofactor tetrahydrobiopterin (BH4), O_2 and Fe^{2+}. In the second step, L-DOPA is decarboxylated to dopamine by aromatic amino acid decarboxylase (AADC, also known as DOPA decarboxylase) in a reaction that uses pyridoxal phosphate (vitamin B6) as a cofactor. The biosynthesis of dopamine and other catecholamines (noradrenaline and adrenaline) is under strong regulatory control. The activity of AADC, for example, is under feedback regulation by the reaction product.

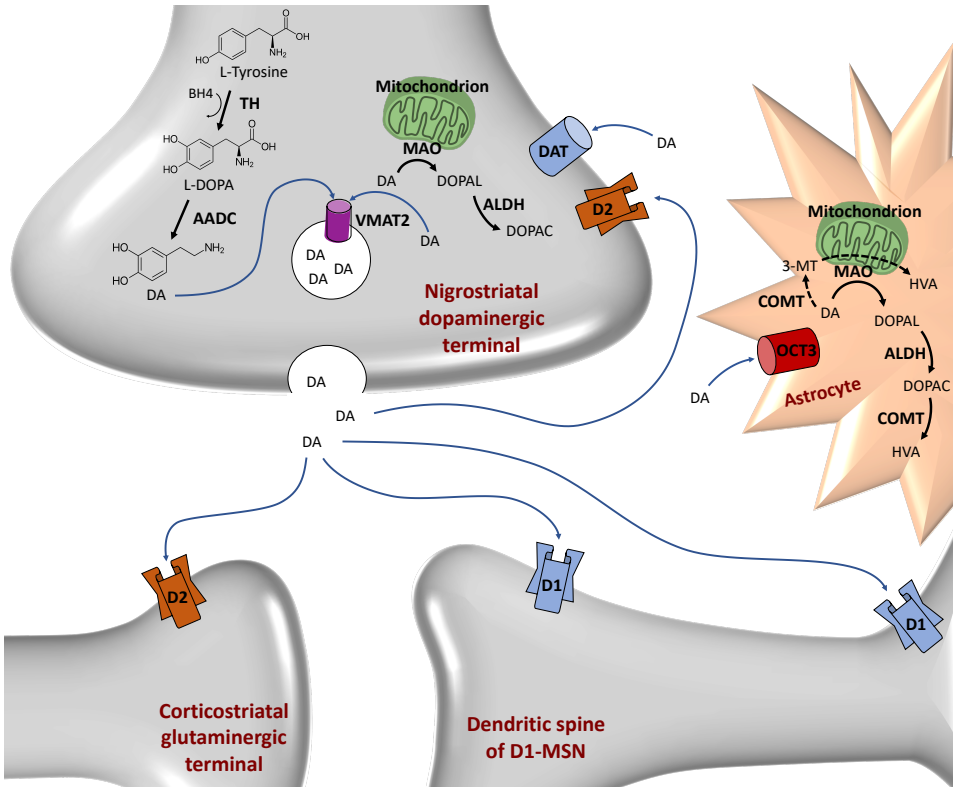

Figure 2.8. Nigrostriatal dopaminergic axon terminal and dopamine lifecycle. Dopamine (DA) synthesis takes place in the cytosol. Tyrosine hydroxylase (TH) hydroxylates L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) using tetrahydrobiopterin (BH4) as a cofactor. L-DOPA is decarboxylated to DA by aromatic amino acid decarboxylase (AADC). DA is sequestered into synaptic vesicles via vesicular monoamine transporter 2 (VMAT2). Upon depolarization of the axon terminal, synaptic vesicles are fused into the presynaptic membrane in a Ca^{2+}-dependent manner resulting in quantal release of DA into the extracellular space. Dopaminergic axons form synaptic contacts on the dendritic spines necks of medium spiny neurons (MSN). Released DA diffuses into perisynaptic sites and activates postsynaptic D1- and D2-like receptors located along the dendritic membrane of MSNs. Presynaptic D2 receptors function as autoreceptors providing feedback inhibition of DA synthesis and release. Dopamine transporter (DAT) is responsible for the neuronal high-affinity reuptake of DA and termination of the signaling, after which DA is recycled back into the synaptic vesicles via VMAT2. DAT is distributed outside of the synaptic membrane. Polyspecific organic cation transporter 3 (OCT3) mediates non-neuronal uptake of DA into surrounding glial cells. In the glia, DA undergoes degradation, where DA is first metabolized to 3,4-dihydroxyphenylacetaldehyde (DOPAL) via monoamine oxidase (MAO). DOPAL is rabidly further converted to 3,4-dihydroxyphenylacetic acid (DOPAC) via aldehyde dehydrogenase (ALDH). Catechol-*O*-methyl transferase (COMT) methylates DOPAC to the main metabolite homovanilic acid (HVA). Alternatively, DA is first metabolized to 3-methoxytyramine (3-MT) via COMT, which is then converted to HVA by MAO and ALDH. Figure drawn by the author.

TH is the rate-limiting enzyme in the biosynthesis of catecholamines (Kumer and Vrana 1996). The regulation of TH expression and activity is strong and versatile. TH mRNA levels are under transcriptional and RNA stability regulation. TH protein undergoes translational control and allosteric modulations (Daubner et al. 2011). Dopamine competes with the cofactor BH4 to bind to the catalytic site of TH (Kumer and Vrana 1996). Thus, high dopamine levels exert feedback inhibition on the enzyme activity. In addition, various kinases can phosphorylate four different serine residues in the N-terminal regulatory domain of TH which results in substantially increased enzyme activity (Dunkley et al. 2004). Reversely, phosphatases can dephosphorylate TH, and therefore function as TH deactivators. Interaction with other proteins, such as DJ-1, alpha-synuclein (α-syn), VMAT2, AADC and BH4 synthesizing guanosine triphosphate cyclohydrolase (GTPCH), controls the stability and activity of TH (Daubner et al. 2011). TH can also undergo ubiquitylation and subsequent proteasomal degradation (Døskeland and Flatmark 2002).

TH-immunolabelling is commonly used to identify dopamine neurons in the ventral midbrain. However, due to the strong regulation of TH expression in response to aging, neuronal insults or altered functional demands, TH-immunoreactivity is not always a reliable marker for identifying viable dopamine neurons (Björklund and Dunnett 2007). Thus, in the absence of TH-immunoreactivity, additional markers such as neuromelanin, DAT, AADC or VMAT2 may be needed to identify cells as functional dopamine neurons.

Interestingly, Nurr1, a transcription factor belonging to the orphan nuclear receptor superfamily, is predominantly expressed in dopamine neurons and essential in the development and maintenance of the midbrain dopaminergic system (Jankovic et al. 2005). Nurr1 regulates the expression of TH, DAT, VMAT2, AADC, GDNF and RET, in other words, all the proteins that are crucial for the normal function of dopamine neurons. Studies in Nurr1 knockout mice have shown that Nurr1 deficiency results in impaired function and increased vulnerability of midbrain dopamine neurons. Nurr1 expression is also decreased in the midbrain samples of PD patients. Thus, it may have a role in the pathogenesis of PD.

Besides the classical TH-dependent pathway, the biosynthesis of dopamine can also follow a cytochrome P450-mediated pathway where decarboxylation of tyrosine to tyramine by AADC precedes the hydroxylation step, in which CYP2D6 converts tyramine to dopamine (Hiroi et al. 1998; Bromek et al. 2011). However, the contribution of this pathway to the total dopamine synthesis seems to be minimal.

After synthesis, dopamine is readily sequestered into synaptic vesicles by VMAT2 (Chaudhry et al. 2008). Vesicular dopamine transport depends on the electrochemical H^+ gradient generated by the vacuolar H^+-ATPase. Inside the vesicles, oxidation-prone dopamine is stabilized by acidic pH which prevents the generation of toxic derivatives in the cytosol (Vergo et al. 2007). In noradrenergic neurons, dopamine is further hydroxylated to noradrenaline by dopamine-β-hydroxylase inside the vesicles (Daubner et al. 2011). Amphetamine and similar psychostimulants deplete dopamine from the synaptic vesicles by collapsing the electrochemical H^+ gradient that drives the VMAT2-mediated dopamine transport (Sulzer and Rayport 1990; Chaudhry et al. 2008). Amphetamines can also inhibit VMAT2 directly.

2.2.7.2 Dopamine release and reuptake

Nigrostriatal dopamine neurons are autonomous pacemakers. They typically exhibit two main activity patterns: Under basal conditions, most dopamine neurons show tonic activity with regularly spaced spikes at 2-5 Hz which results in tonic dopamine release in the striatum (Grace and Bunney 1984; Hyland et al. 2002). Rewarding events or other stimuli can trigger brief higher-frequency bursts of approximately two to six action potentials at 15-30 Hz which evoke transient dopamine overflow from the synaptic vesicles. Aversive events, on the other hand, transiently decrease nigrostriatal dopaminergic activity. These transient changes in striatal dopamine release regulate motivation and movement initiation (Klaus et al. 2019).

In response to action potentials arriving to an axon terminal, depolarization opens voltage-gated Ca^{2+} channels allowing rapid influx of Ca^{2+}. Inside the axon terminal, Ca^{2+} induces conformational changes in synaptic soluble N-ethylmaleimide–sensitive factor attachment protein receptor (SNARE)–complex proteins which eventually lead to exocytotic release of synaptic vesicles filled with dopamine (Sulzer et al. 2016). Using electrochemical cytometry, it has been estimated that the average number of dopamine molecules per vesicle is 33 000 in striatal axon terminals of healthy mice, i.e. the quantal size of dopamine release is 33 000 molecules (Omiatek et al. 2013). Under basal conditions, the extracellular dopamine concentration resulting from tonic neuronal activity is in the range of 10-30 nM (Gonon and Buda 1985; Venton et al. 2003). It is high enough to tonically activate the high-affinity pre- and postsynaptic D2 receptors.

Some of the midbrain dopamine neurons, that express the vesicular glutamate transporter 2 (VGLUT2), can co-release glutamate in addition to dopamine (Chuhma et al. 2014; Hnasko et al. 2010; Stuber et al. 2010; Tecuapetla et al. 2010). Dopamine neuron -evoked AMPA and NMDA receptor activation have been reported in MSNs and cholinergic interneurons of the NAcc. Recently, SNpc dopamine neuron inputs were shown to drive pauses in the firing of DMS and bursts in the firing DLS cholinergic interneurons (Cai and Ford 2018). The pauses were mediated by D2 receptors while the bursts were driven by glutamate co-release and activation of excitatory metabotropic glutamate receptors (mGluRs). Also, rapidly after dopaminergic activation, firing in both direct- and indirect MSNs is inhibited by co-release of GABA from a subset of dopaminergic axon terminals (Tritsch et al. 2012; 2014; Chuhma et al. 2014). The co-release of GABA seems to require its cellular uptake by membrane GABA transporters (mGAT1 and mGAT4) and loading into dopaminergic vesicles by VMAT2.

α-Syn is a 140-amino acid long presynaptic protein that interacts with negatively charged phospholipid membranes through its N-terminus, is ubiquitously expressed throughout the nervous system and is prone for aggregation in PD (Burré et al. 2013). Although the precise function of α-syn is not known, it seems to be an essential negative regulator of synaptic neurotransmission and activity-dependent dopamine release (Abeliovich et al. 2000; Larsen et al. 2006; Nemani et al. 2010). A growing body of evidence suggests a role for α-syn in the maintenance of synaptic vesicle pools and/or as an auxiliary chaperone required for normal SNARE-complex assembly (Burré et al. 2010; Cabin et al. 2002; Chandra et al. 2005; Murphy et al. 2000; Snead and Eliezer 2014). Transgenic mice overexpressing human wild-type α-syn show

selective alteration in the distribution of dopaminergic synaptic vesicles and deficits in dopamine release from the nigrostriatal axon terminals (Janezic et al. 2013). This early-onset phenotype is followed by age-dependent loss of dopamine neurons, reduced neuronal firing rate and motor impairments.

Other neurotransmitters can also regulate axonal dopamine release. In the dorsal striatum, glutamate spillover from its synapses can reduce dopamine release by acting on presynaptic mGluR1 on dopaminergic axon terminals (Zhang and Sulzer 2003). GABA has been shown to decrease dopamine release via presynaptic $GABA_B$ receptors (Charara et al. 1999; Smolders et al. 1995). In the NAcc, kappa and delta opioid receptors seem to be able to decrease dopamine release via a presynaptic mechanism (Schlösser et al. 1995; Svingos et al. 1999; 2001).

Dopamine released into the extracellular space can interact with postsynaptic dopamine receptors and presynaptic autoreceptors (Figure 2.8). The postsynaptic receptors are mostly located at distant extrasynaptic sites, often with a higher density in the perisynaptic zone of the dendritic spines of striatal MSNs (Hersch et al. 1995; Yung et al. 1995; Caillé et al. 1996). Therefore, dopamine has to spillover from the release sites and diffuse rather long distances to reach its receptors (Pickel et al. 1996). This kind of “volume transmission” is characteristic for monoamine transmitters and can modulate not only neuronal activity in larger brain areas, but also neuron-glia interactions (Fuxe et al. 2015; Taber and Hurley 2014). Diffusion-based dilution is also the main factor eventually terminating the dopamine signaling in most brain areas (Cragg and Rice 2004; Rice and Cragg 2008).

Another important factor contributing to the termination of the dopamine signaling and maintaining dopamine homeostasis is its effective reuptake from the extracellular space primarily via DAT (Meiser et al. 2013). DAT is rarely expressed on the active zone of a synapse. Instead, the dopamine reuptake sites are distributed along the membrane of dopaminergic fibers where they effectively restrict the extracellular diffusion of larger dopamine transients (Nirenberg et al. 1996; Pickel et al. 1996; Hersch et al. 1997). After release, dopamine can either be recycled back into dopaminergic axon terminals by DAT, or it can be degraded after non-neuronal uptake into surrounding glial cells by organic cation transporter 3 (OCT3) (so called uptake 2). OCT3 is a low-affinity and high-capacity transporter for monoamines widely expressed in neurons, astrocytes, microglia and oligodendrocytes (Eisenhofer 2001; Gasser 2019). It contributes to the clearance of monoamines after synaptic release and restricts their spread. In the striatum, DAT-mediated neuronal uptake is primarily responsible for the clearance of extracellular dopamine. Substrate-induced and PKC-dependent trafficking of DAT away from the cell surface is the predominant mechanism to regulate DAT function (Gulley and Zahniser 2003). For example, amphetamine typically induces DAT internalization. In addition, presynaptic D2 receptors mediate the upregulation of DAT activity. Neuronal uptake of dopamine is followed by its sequestration back into the synaptic vesicles via VMAT2.

2.2.7.3 Dopamine degradation

Following the uptake 2 by glial cells, dopamine is readily degraded by MAO and catechol-*O*-methyl transferase (COMT) (Meiser et al. 2013). There are two separate genes coding two isoforms of MAO, MAO-A and MAO-B, which are both found in the CNS. MAO is localized in the outer membrane of mitochondria and expressed in neurons, astrocytes and microglia. MAO catalyzes oxidative deamination of dopamine to a reactive intermediate product 3,4-dihydroxyphenylacetaldehyde (DOPAL) generating hydrogen peroxide as a side product (Eisenhofer et al. 2004). DOPAL is further metabolized predominately by aldehyde dehydrogenase to form 3,4-dihydroxyphenylacetic acid (DOPAC), or by aldehyde reductase to form 3,4-dihydroxyphenylethanol (DOPET), both of which are more stable metabolites. In neurons, dopamine that is leaking from the synaptic vesicles into the cytosol, is degraded by MAO and aldehyde dehydrogenase (Meiser et al. 2013). Notably, there are species specific differences in the affinities of MAO-A and MAO-B: in human, dopamine is mostly oxidized by MAO-B, but in rats by MAO-A.

COMT has two splicing isoforms encoded by one gene, soluble S-COMT and membrane-bound MB-COMT. Their role is to transfer a methyl group from S-adenosylmethionine to one of the two catechol hydroxyl groups (Guldberg and Marsden 1975; Männistö and Kaakkola 1999). This 3-O-methylation of DOPAC is Mg^{2+} -dependent and leads to the formation of homovanilic acid (HVA) which is the main degradation product of dopamine and ultimately secreted into urine. In an alternative degradation pathway, dopamine is first methylated to 3-methoxytyramine (3-MT) by COMT, which is then oxidized to HVA by MAO and aldehyde dehydrogenase (Meiser et al. 2013). There is no significant COMT activity in presynaptic axon terminals but some activity in postsynaptic dendrites. The main COMT-activity seems to reside in astrocytes and microglial cells (Myöhänen et al. 2010).

Dopamine and its metabolites can also undergo phase II conjugation reactions before excretion, although the conjugation reactions seem to play only a minor role in dopamine metabolism in the CNS (Uutela et al. 2009). O-Sulfatation of dopamine or its metabolites is catalyzed by phenol sulfotransferases (Buu et al. 1981; Swahn and Wiesel 1976), and O-glucuronidation by uridine diphosphoglucuronosyl-transferases (Wang et al. 1983; Uutela et al. 2009).

The metabolism of dopamine inflicts constant oxidative stress on dopaminergic neurons that might be one predisposing factor for the specific neuronal vulnerability in PD (Meiser et al. 2013). High dopamine content exposes to vesicular leakage of dopamine into the cytosol. The degradation of cytosolic dopamine generates reactive oxygen species (ROS). As described above, oxidative deamination by MAO generates hydrogen peroxide that is a major source of oxidative stress in dopaminergic neurons. Besides this, the electron-rich catechol moiety of dopamine is also prone to oxidation if exposed to the neutral pH of the cytosol. Dopamine and L-DOPA can also be enzymatically oxidized to form highly reactive ortho-quinones dopamine-o-quinone and dopaquinone, respectively (Sulzer and Zecca 1999). Both, quinones and ROS, are apt to react unspecifically with cellular components and perturb their functionality. Oxidative stress and

changes in cellular functions can trigger neuroinflammation which is a characteristic feature in PD and can contribute to the degeneration of dopamine neurons.

2.3 Parkinson's disease

The first coherent descriptions of a clinical syndrome resembling PD can be found from ancient Indian medical literature dating back to 300 BC (later known as Kampavata) (Ovallath and Deepa 2013). In Western medicine, a British physician James Parkinson provided the first sound picture of PD with all the characteristic motor symptoms in his famous monograph An Essay on the Shaking Palsy published in 1817 (Parkinson 2002). Almost 150 years later, striatal dopamine depletion was associated with PD (Ehringer and Hornykiewicz 1960), and the effectiveness of L-DOPA in alleviating PD symptoms was demonstrated and brought into clinical practice (Birkmayer and Hornykiewicz 1961; Cotzias et al. 1969).

Although the emerge of motor symptoms is typically diagnostic, PD is not solely a movement disorder but associated with numerous debilitating non-motor symptoms (Kalia and Lang 2015). Until today, the most urgent unmet medical need in PD is a disease-modifying therapy that could halt or slow the progression of the disease and restore the neuronal circuits that have already been degenerated by the time of the diagnosis. Accumulating preclinical evidence for the neurorestorative properties of several NTFs seems convincing (Paul and Sullivan 2018). However, to date, none of them has translated into clinical use, although several clinical trials have been conducted and some are currently underway. This calls for new approaches to harness the regenerative potential of neuroplasticity in the brain.

Apart from the lack of a cure and incomplete understanding of the pathogenesis, a particular clinical challenge of PD management is that there are no biomarkers which would allow definitive diagnosis at early stages of the disease. Better diagnostic tools would also facilitate better distinction between different subtypes of PD enabling more effective personalized therapies.

2.3.1 Epidemiology and risk factors

PD is the second most common neurodegenerative disease after AD, and the most common movement disorder (Hebert et al. 2013; Marras et al. 2018). It is estimated to affect more than 6 million people worldwide causing over 200 000 deaths per year (Dorsey et al. 2018). The incidence of PD sharply increases with age which is the biggest risk factor for the disease (Driver et al. 2009; Pringsheim et al. 2014). On average 1% of people aged over 60 years lives with the disease, and the prevalence increases to about 2-4% in individuals over the age of 80 (de Lau and Breteler 2006; Pringsheim et al. 2014). With the overall aging of the population, PD is expected to impose an increasing socio-economic burden on societies. Men are approximately 1.5 times more likely to be afflicted than women (de Lau and Breteler 2006). The risk of developing sporadic PD is multifactorial involving a complex interplay between normal process of aging, genetic susceptibility, epigenetic mechanisms and environmental risk factors (Pang et al. 2019). The fundamental cause of the disease remains unknown in the large majority of the cases.

2.3.1.1 Environmental risk factors

Several comprehensive meta-analyses have identified pesticide, herbicide and solvent exposure, traumatic head injury, consumption of dairy products, rural living, beta-blocker use, agricultural occupation and well-water drinking as environmental risk factors that significantly increase the risk of PD (Noyce et al. 2012; Pezzoli and Cereda 2013; Ascherio and Schwarzschild 2016; Yang et al. 2017b; Pang et al. 2019). Environmental factors found to have a significant negative association with PD are tobacco use (cigarette smoking or smokeless tobacco) and caffeine consumption. Importantly, physical activity has been reported to reduce the risk of PD, and this potential protective effect has been related to the ability of exercise to stimulate antioxidant defense, angiogenesis and NTF expression (Cohen et al. 2003; Zigmond and Smeyne 2014; Bellou et al. 2016).

2.3.1.2 Genetic susceptibility

Only 5-10% of PD patients suffer from a familial form of the disease (Kalinderi et al. 2016). To date, more than 20 causal genes and 90 independent genetic risk variants for PD have been identified (Blauwendraat et al. 2020). The gene encoding α-syn, *SNCA*, was the first one found to cause monogenic PD (Polymeropoulos et al. 1997). *SNCA* missense mutations and multiplications of the gene locus cause autosomal dominant form of the disease by increasing the tendency of α-syn to misfold and form aggregates (Deng et al. 2018). The most frequent cause for late-onset and autosomal dominant PD are missense gain-of-function mutations in *LRRK2*-gene (Paisán-Ruíz et al. 2004; Zimprich et al. 2004; West et al. 2005; Deng et al. 2018; Pang et al. 2019). *LRRK2* mutations and elevated kinase activity can also be found in some sporadic cases (Di Maio et al. 2018; Simón-Sánchez et al. 2009). *LRRK2* encodes a large multidomain protein called leucine-rich repeat kinase 2 which is involved in multiple cellular processes, e.g. neurite outgrowth, synaptogenesis, vesicular trafficking, autophagy and protein synthesis (Dzamko and Halliday 2012; Martin et al. 2014).

Loss-of-function mutations in *PRKN*, *PINK1* and *DJ-1* are the most common examples of genes accounting for autosomal recessive forms of PD (Pang et al. 2019). Recessively inherited mutations in these genes are often associated with early-onset PD (Schrag and Schott 2006). Parkin (encoded by *PRKN*), PINK1 and DJ-1 are implicated in mitochondrial function, dynamics and quality control (McCoy and Cookson 2012). Mutations in *GBA*, which encodes a lysosomal enzyme β-glucocerebrosidase, form another major risk factor for early-onset PD and cause a more severe phenotype of PD with increased risk for dementia and reduced survival (Cilia et al. 2016; Lwin et al. 2004; Sidransky and Lopez 2012). Apart from the abovementioned typical examples, advances in human genetics and bioinformatics have identified various other PD-causing genes and risk loci as reviewed in (Deng et al. 2018) and (Blauwendraat et al. 2020).

2.3.2 Pathology of Parkinson's disease

2.3.2.1 Neurodegeneration

PD is a complex neurodegenerative disorder. The main pathological feature is progressive loss of dopamine neurons within the SNpc (Kalia and Lang 2015). The neuronal loss and the accompanying denervation of the nigrostriatal pathway leads to decreased dopamine release in the dorsal striatum (Figure 2.9.A and C). Striatal dopamine depletion disrupts the equilibrium between striatonigral and striatopallidal pathway MSNs resulting in increased activation of the GABAergic output nuclei of the basal ganglia (Mallet et al. 2006; McGregor and Nelson 2019). The outcome of these pathological changes is reduced activation of the motor cortex, and ultimately, the appearance of the cardinal parkinsonian motor symptoms (see below chapter 2.3.3).

When PD is diagnosed, a substantial proportion of dopamine neurons in the SNpc has already died. It has been estimated that at the onset of the motor symptom there is approximately 30% loss of dopaminergic cell bodies in the SNpc and 50-70% loss of striatal dopaminergic terminals in comparison to age-matched controls (Burke and O'Malley 2013). Thus, the molecular and cellular neuropathology is likely to start years or decades before the diagnosis. Over the course of the disease, the neurodegeneration continues progressing and results in 60-80% loss of nigral dopamine neurons and almost complete loss of striatal fibers at the time of death (Burke and O'Malley 2013; Kordower et al. 2013). The greater loss of striatal fibers as compared to the loss of neuronal bodies in the SNpc suggests that in PD the neurodegeneration occurs in a "dying-back" fashion with the distal axons deteriorating first and the cell bodies dying only months or years later. In addition, the substantial neurodegeneration observed at the time of the diagnosis suggests a prolonged preclinical stage. These two characteristics offer a clear therapeutic window for disease-modifying interventions.

Axonal degeneration of the nigrostriatal dopamine neurons is proposed to be an early pathological process and mediated by different mechanisms than those responsible for the degeneration of the cell bodies (Cheng et al. 2010; Tagliaferro and Burke 2016; O'Keeffe and Sullivan 2018). Axonopathy is accompanied with disturbances in axonal transport which is another important feature in the early-stage PD pathology (Chu et al. 2012; De Vos et al. 2008). The lack of proper axonal transport can interfere with many neuronal functions crucial, for example, for synaptic communication. Accumulation of α-syn inclusions in the axons in the early stages of the disease has been shown to hamper the retrograde transport of signaling endosomes from axon terminals to the cell bodies (Volpicelli-Daley et al. 2014). Importantly, this may have implications for neuroprotective actions of striatally secreted NTFs and reduce the effectiveness of interventional neurotrophic therapeutics administered into the striatum (Bartus et al. 2011).

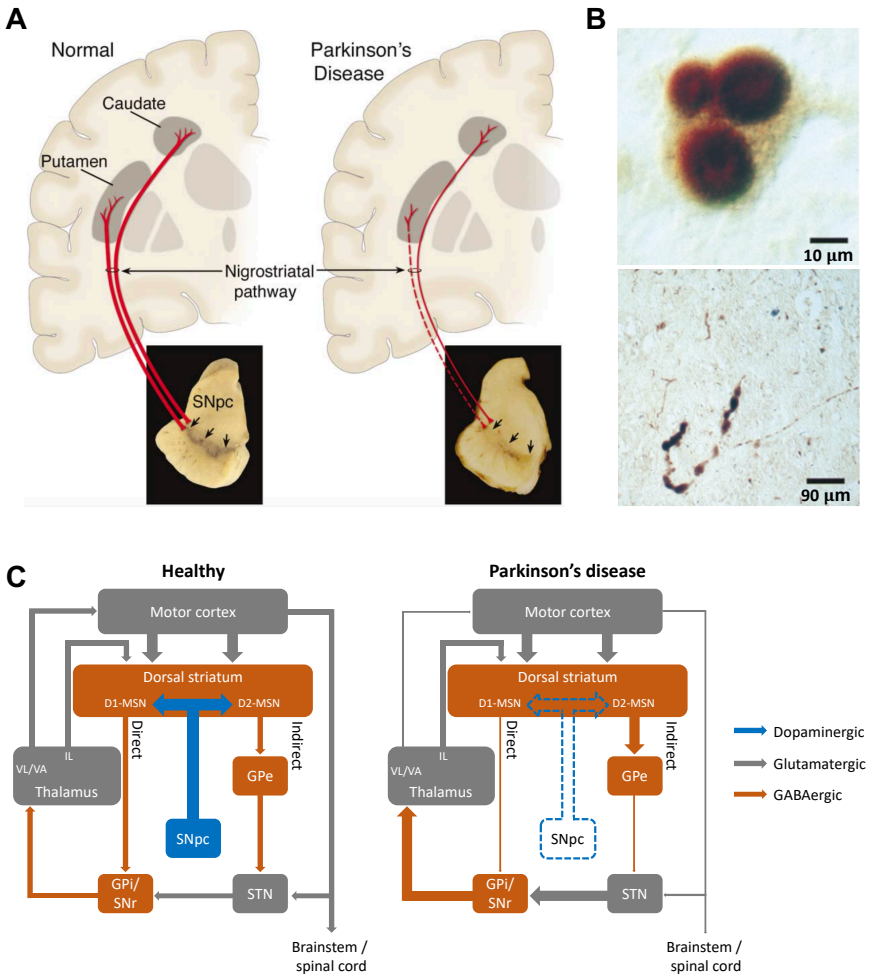

Figure 2.9. Neuropathology of Parkinson's disease (PD). (**A**) Left panel: The cell bodies of dopamine neurons are located in the substantia nigra pars compacta (SNpc). In intact nigrostriatal pathway, neuromelanin in dopamine neurons produces the dark pigmentation of the SNpc as shown in the photograph of the brainstem transverse section (black arrows). Dopamine neurons project to the dorsal striatum (i.e. putamen and caudate nucleus) as illustrated with thick solid red lines. Righ panel: Degeneration of the nigrostriatal pathway is the pathological hallmark of PD. The loss of neuromelanin-containing dopamine neurons causes depigmentation of the SNpc (black arrows). The loss of dopaminergic projections to the putamen (dashed red line; prominent loss) and the caudate nucleus (thin solid red line; less prominent loss) results in dopamine depletion in the dorsal striatum. Reproduced with permission from Elsevier: Neuron, Dauer and Przedborski (2003), © Cell Press 2003 (**B**) Intracellular Lewy pathology in SNpc specimens from a PD patient. Upper panel: Nerve cell body with three Lewy body inclusions that are double-stained for alpha-synuclein and ubiquitin. Lower panel: Lewy neurites in neuronal processes double-stained for alpha-synuclein and ubiquitin. Images adopted from Spillantini et al. (1998), © 1998 by The National Academy of Sciences (**C**) Classical (simplified) "Box and Arrow model" of cortico-basal ganglia-thalamocortical circuitry depicting the essential neuronal pathways in the healthy brain (left panel) and PD affected brain (right panel). In PD, the excitability of the direct and indirect pathways shifts in opposite directions following the dopamine depletion in the dorsal striatum; the excitability of D1 receptor expressing medium spiny neurons (D1-MSN) reduces whereas the excitability of D2-MSN increases. These changes contribute to the increased firing of the output nuclei internal globus pallidus (GPi) and substantia nigra pars reticulata (SNr). The resultant suppression of the motor thalamus leads to the decreased activation of the motor cortex. VL - ventral lateral nucleus of the thalamus; VA - ventral anterior nucleus of the thalamus; IL - intralaminar thalamic nuclei; STN - subthalamic nucleus; GPe - external globus pallidus. Figure drawn by the author, inspired by Albin et al. (1989).

The neuropathological processes are not confined exclusively to dopaminergic neurons but affect several non-dopaminergic neurotransmitter systems too. Histopathological, biochemical and imaging findings in PD patients have demonstrated the concomitant degeneration of serotonin, noradrenaline, acetylcholine and various neuropeptides containing neurons (Halliday et al. 1990; Gesi et al. 2000; Hirsch et al. 2003; Kish et al. 2008). Widespread neuronal loss can be found in several non-dopaminergic nuclei including the locus coereleus, the nucleus basalis of Meynert, the dorsal motor nucleus of the vagus, the pedunculopontine nucleus, the raphe nuclei, amygdala and hypothalamus (Dickson 2012; Giguère et al. 2018). Dysfunctions in these systems are likely to contribute to the manifestation of non-motor symptoms of PD such as sleep disorders, depression and cognitive impairment (see chapter 2.3.3).

2.3.2.2 Pathological protein accumulation

The other pathological hallmark of PD is the presence of cytoplasmic fibrillar protein inclusions called Lewy bodies (within cell bodies) and Lewy neurites (mostly in dystrophic neuronal processes) (Goedert et al. 2013) (Figure 2.9.B). Lewy inclusions are composed of numerous cellular proteins, the most abundant being misfolded α-syn, together with membranous material originating from vesicles and fragmented organelles (Shahmoradian et al. 2019; Spillantini et al. 1998, 1997; Wakabayashi et al. 2013; Xia et al. 2008). Misfolded α-syn has the propensity to acquire a β-sheet-rich structure and form insoluble amyloid fibrils. Several post-translational modifications, including phosphorylation at serine 129, nitration and C-terminal truncation, have been proposed to be responsible for the conformational changes that contribute to the fibrillization of α-syn (Barrett and Greenamyre 2015). A recent study by Mahul-Mellier et al. (2020) suggests that Lewy body formation involves a complex interplay between α-syn fibrillization, post-translational modifications and interactions between α-syn aggregates and membranous organelles. They argued that different stages of Lewy body formation, rather than simply α-syn fibril formation, lead to molecular events that contribute to neuronal dysfunction and degeneration. Until today, however, the causal role of the Lewy pathology in the neuronal loss has not been confirmed (Kalia and Lang 2015). Clinical PD can also occur in the absence of Lewy pathology, particularly in certain monogenic forms of the disease such as most of the *PRKN* mutation -related cases and a proportion of patients with *LRRK2* mutations (Poulopoulos et al. 2012; Doherty et al. 2013; Kalia et al. 2015).

As intermediates of the aggregation process α-syn forms soluble oligomers and protofibrils that are proposed to be the most neurotoxic species potentially damaging cell membranes (Kalia et al. 2013; Karpinar et al. 2009; Winner et al. 2011). Some types of α-syn oligomers may increase membrane permeability via a pore-forming mechanism which leads to Ca^{2+} influx and disruption cellular ion homeostasis (Angelova et al. 2016; Danzer et al. 2007). Notably, misfolded α-syn loses its physiological functionality in nerve terminals which leads to synaptic dysfunction characterized by compromised neurotransmitter release and enlarged synaptic vesicles (Scott et al. 2010).

A number of cellular mechanisms are implicated in PD pathogenesis, including mitochondrial dysfunction, oxidative stress, ER stress, defective protein degradation systems, impaired

intracellular Ca^{2+} homeostasis and neuroinflammation, as described in the following chapters. However, their mechanistic details and interrelations have yet to be elucidated.

2.3.2.3 Braak staging system and Lewy pathology spreading

Lewy pathology spreads and afflicts multiple neuroanatomical areas in the brain. In 2003, Braak and colleagues introduced a staging system of Lewy pathology propagation based on semiquantitative assessment of Lewy body inclusions in *postmortem* autopsy samples of incidental and symptomatic sporadic PD cases (Braak et al. 2003). This hypothesis proposes that Lewy pathology spreads in an ascending direction throughout the brain in a sequence of six stages from medullary and olfactory nuclei towards the cortical areas as described in Figure 2.10.A. Generally, the staging system seems to apply to the majority of PD cases studied *postmortem* making it widely accepted (Dickson et al. 2010; Postuma et al. 2012). However, some studies have shown that not all cases fit to the hypothesis questioning its predictive validity and underpinning the heterogenous nature of PD (Kalaitzakis et al. 2008; Parkkinen et al. 2008; Jellinger 2009; Halliday and McCann 2010).

The Braak hypothesis was later revised to postulate that the initial event in sporadic PD is the access of α-syn pathology to the brain through nasal and intestinal mucosal sites (Braak et al. 2003b; Braak et al. 2006; Hawkes et al. 2007; Hawkes et al. 2009). According to this "dual-hit hypothesis" α-syn pathology enters the brain from the olfactory bulb via anterograde progression into the temporal lobe, as well as from the enteric plexuses via transsynaptic transmission and retrograde transport along the vagus nerve into the medulla and pons. Indeed, Lewy pathology is not restricted to the brain in PD; α-syn immunoreactive inclusions have a widespread distribution throughout the spinal cord and PNS as well as multiple peripheral organs including skin, heart, submandibular gland and gastrointestinal system (Beach et al. 2010; Bloch et al. 2006; Wakabayashi and Takahashi 1997). This lends support to the hypothesis that the disease originates from the periphery. Interestingly, an epidemiological study showed that a full truncal vagotomy was associated with a decreased risk of subsequent PD supporting the role of the vagus as a gateway to the brain (Perez-Pardo et al. 2017; Svensson et al. 2015). The dual-hit hypothesis goes well together with hyposmia and constipation being prevalent prodromal symptoms in PD (see chapter 2.3.3).

The Braak hypothesis gained support from the observations that α-syn aggregates can transfer between neurons. Findings of Lewy bodies in fetal dopamine neurons grafted into the striatum of PD patients showed that Lewy pathology can spread from host to grafted cells, and triggered the idea that the propagation of Lewy pathology is mediated by a prion-like transmission of α-syn between neurons (Chu and Kordower 2010; Kordower et al. 2008; Li et al. 2008; Visanji et al. 2013). These findings have been confirmed in several *in vivo* experiments where intracerebral inoculation of synthetic recombinant α-syn fibrils or Lewy body extracts from PD brains induced spreading of α-syn pathology to anatomically interconnected brain areas and subsequent neurodegeneration (Luk et al. 2012; Recasens et al. 2014; Chu et al. 2019; Henderson et al. 2019). Indeed, mounting evidence has demonstrated the cell-to-cell transmission of small α-syn aggregates, their ability to seed further aggregation via recruitment of endogenous host α-syn in the recipient cells and their

bidirectional axonal transport (reviewed in Brundin et al. 2016; Killinger and Kordower 2019). The molecular mechanisms of α-syn release to the extracellular space and uptake by neighboring cells are not fully elucidated and they may vary between different α-syn species and conditions (Tyson et al. 2016). Data from cell culture models suggest that oligomeric and fibrillar α-syn can be released via exocytosis in a process that is enhanced by cellular stress, or through uncontrolled leakage upon cell death. The uptake of α-syn is believed to be mediated by receptor mediated endocytosis (Karpowicz et al. 2019).

Lewy pathology can also coexist with other proteinaceous inclusions, such as cytoplasmic tau-containing neurofibrillary tangles and extracellular β-amyloid (Aβ) plaques, suggesting interactions between pathogenic mechanisms of different proteinopathies (Jellinger 2012). Tau and Aβ inclusions have been detected *postmortem* in the cortex of up to half of cognitively impaired or demented PD patients (Compta et al. 2011; Irwin et al. 2013). Thus, comorbidity with AD-type pathologies seems to be associated with the cognitive decline in PD.

2.3.2.4 Dysfunctional protein degradation systems

Abnormal proteins are removed from cells via two main protein clearance systems: ubiquitin-proteasome system (UPS) and autophagy-lysosome pathway (ALP) (Rubinsztein 2006). Both UPS and ALP are responsible for the clearance of intracellular α-syn (Rocha et al. 2018). Hence, dysfunction in either of these proteolytic machineries is a potential pathogenic mechanism in PD (Olanow and McNaught 2006; Pan et al. 2008; Ebrahimi-Fakhari et al. 2012; Xilouri et al. 2013; Lehtonen et al. 2019). Upon impaired degradation, misfolded α-syn and other defective proteins accumulate in the cytoplasm contributing to their aggregation, perturbed cellular functions and cytotoxicity. Reciprocally, accumulating aberrant α-syn species may further aggravate the dysfunction of UPS and ALP forming a vicious feedback loop that leads to neuronal death. The activity of both UPS and ALP declines with aging which may play a role in the neurodegenerative process (Xilouri et al. 2013).

The first evidence of the UPS abnormalities in PD was provided by *postmortem* studies of the SNpc where the proteasomal catalytic activity was found the be significantly reduced when compared to healthy brains (McNaught and Jenner 2001). Apart from the diminished activity, lower expression of different components involved in the normal function of UPS was also identified in the SNpc of PD brains (McNaught et al. 2002, 2003). Additional evidence is provided by genetic studies. Two genes linked to monogenic forms of PD are involved in UPS function: *PRKN* encodes parkin, an E3 ubiquitin ligase, which ubiquitinates substrate proteins and is needed for normal proteasome activation. PD-linked mutations in *PRKN* abolish this function which is thought to lead to the manifestation of the disease (Kitada et al. 1998; Um et al. 2010). A missense mutation in the gene encoding ubiquitin C-terminal hydrolase L1 (UCH-L1; a neuron-specific ubiquitin recycling enzyme) causes partial loss of the enzymatic activity and has been linked to increased risk of familial PD due to impaired de-ubiquitination and negative regulation of UPS (Leroy et al. 1998; Nishikawa et al. 2003).

Animal models of PD also provide support to the notion that impaired UPS is an important etiopathogenic factor in PD. In wild-type rats, selective proteasome inhibition with an intranigral lactacystin injection caused motor impairment, dose-dependent degeneration of nigrostriatal dopamine neurons and cytoplasmic accumulation of α-syn immunoreactive protein inclusions (McNaught et al. 2002b). In transgenic mice, depletion of 26S proteasomes using a conditional knockout approach led to extensive neurodegeneration of the nigrostriatal pathway and forebrain regions and appearance of intraneuronal Lewy body -like inclusions (Bedford et al. 2008).

Similar to the findings in UPS system, various ALP-related components are differently expressed or dysfunctional in PD. For example, in *postmortem* examinations of the SNpc, chaperone-mediated autophagy-related proteins, such as lysosomal membrane receptors LAMP1 and LAMP2A and heat-shock proteins 70 and 73, were found to be decreased in PD brains as compared to age-matched controls (Alvarez-Erviti et al. 2010; Chu et al. 2009). Several genes linked to ALP have also been associated with PD (Gan-Or et al. 2015). For example, mutant LRRK2 interferes with ALP leading to retardation of α-syn degradation and, as a consequence, its accumulation (Yue and Yang 2013). Additionally, loss-of-function mutations in the *GBA* gene, which encodes the lysosomal enzyme glucocerebrosidase, are strong genetic risk factors for PD suggesting a role for dysfunctional ALP in the pathogenesis of PD (Sidransky and Lopez 2012).

2.3.2.5 Neuroinflammation

Neuroinflammation, with reactive astrocytosis and microgliosis, is strongly implicated in the pathogenesis of PD. Upon immune response, the morphology of microglia changes from a resting state ramified shape to an amoeboid profile (Kim and Joh 2006; Brück et al. 2016). This morphological change is accompanied with alterations in the expression of cell surface receptors and increased release of nitric oxide, ROS and cytokines. Some of the secreted factors are neuroprotective, such as GDNF and BDNF, but the majority are proinflammatory such as TNFα and IL-1β. Thus, reactive microglia amplify neuroinflammatory response and trigger oxidative damage and cytokine-receptor-mediated apoptosis in surrounding cells. This might contribute to the neurodegeneration in PD. On the other hand, reactive microglia can phagocyte cell debris and pathological protein aggregates, thus promoting neuroprotection.

Increased microglia and complement activation, T-cell infiltration and proinflammatory cytokine levels have been detected *postmortem* in the SNpc and striatum specimens from PD patients as compared to age-matched healthy subjects (McGeer et al. 1988; Hunot et al. 1999; Loeffler et al. 2006; Hirsch and Hunot 2009). Neuroimaging studies using positron emission tomography (PET) with a radiotracer for reactive microglia (^{11}C-PK11195) has demonstrated increased and stable microglial activation in the brainstem, basal ganglia and several cortical regions in patients with early-stage PD (Edison et al. 2013; Gerhard et al. 2006; Ouchi et al. 2005). Genetic studies have also identified a single-nucleotide polymorphism within the human leucocyte antigen (HLA) class II region that is associated with increased risk of developing PD, suggesting an immune system-related susceptibility to PD (Hamza et al. 2010; Nalls et al. 2014; Saiki et al. 2010).

Intriguingly, serum immune marker profile seems to be predictive of disease progression in PD patients. Elevated proinflammatory and lower anti-inflammatory marker levels were associated with faster progression of motor symptom and worse cognitive performance (Williams-Gray et al. 2016). In line with this, epidemiological studies have found a potential protective effect of regular non-steroidal anti-inflammatory drug use on the risk of PD (Gagne and Power 2010; Gao et al. 2011).

Although one can consider neuroinflammation to be a secondary phenomenon, accumulating evidence suggests that inflammatory processes *per se* have a contributing role in the pathogenesis of PD. For example, it has been demonstrated in 6-OHDA and MPTP mouse models of PD, that microglial-induced inflammatory events play a role in the neurotoxic effects on nigrostriatal dopamine neurons (He et al. 2001; Wu et al. 2002). In these studies, inhibition of microglia activation with minocycline mitigated dopaminergic cell death in the SNpc and reduced the level of IL-1β. Regardless of whether neuroinflammation has a causal role in neurodegeneration or it is a consequence of neuronal damage, it is clear that the engagement of immune system can exacerbate neuronal dysfunction in PD.

A strong body of evidence suggests that α-syn can directly activate microglia in the CNS and initiate inflammatory response (Rocha et al. 2018). For example, overexpression of human wild-type α-syn resulted in early microglial activation in the SNpc of transgenic mice (Su et al. 2008). In primary culture models, extracellular α-syn triggered microglial activation and increased the expression of proinflammatory cytokines presumably in a receptor mediated manner (Zhang et al. 2005; Klegeris et al. 2008; Su et al. 2008). α-Syn has also been shown to interact with astrocytes via Toll-like receptor 4 inducing nuclear translocation of NF-κB and subsequent astrocyte activation with increased expression of proinflammatory cytokines (Rannikko et al. 2015; Chavarría et al. 2018).

2.3.2.6 Mitochondrial dysfunction, oxidative stress and ER stress

Mitochondrial dysfunction is closely related to sporadic and familial forms of PD (Moon and Paek 2015; Ryan et al. 2015). It can contribute to dopaminergic cell death due to cellular energy depletion, impaired calcium homeostasis, production of free radicals and increased oxidative stress. Early evidence arose from a group of young drug addicts in California who developed severe parkinsonian symptoms after self-administering a synthetic heroin (pethidine analogue) which was contaminated with a synthesis by-product MPTP (Langston et al. 1999, 1983). Later, *postmortem* examination of these individuals revealed that MPTP had selectively destroyed dopamine neurons in the SNpc. MPTP, when oxidized to MPP+, is taken up by dopamine neurons and leads to the inhibition of mitochondrial complex I which is a vital component in the electron transport chain (Nicklas et al. 1985). Other toxins and pesticides that interfere with mitochondrial complex I, like rotenone and paraquat, are also linked to the increased risk of PD (Tanner et al. 2011). Furthermore, in idiopathic PD cases, *postmortem* studies of the SNpc showed deficiency in mitochondrial complex I providing another direct indication of the mitochondrial dysfunction in PD (Schapira et al. 1989).

Importantly, several genes associated with monogenic forms of PD play a role in mitochondrial homeostasis. For example, PINK1 and parkin are the key regulators of mitophagy that is a process to dispose damaged mitochondria (Pickrell and Youle 2015; Ryan et al. 2015). The function of DJ-1 is less well characterized, but it seems to protect mitochondria from oxidative stress and be involved in maintaining mitochondrial homeostasis. Interestingly, α-syn itself is known to interfere with mitochondrial bioenergetics. In the striatum and SNpc autopsy samples from PD subjects, α-syn was shown to accumulate in the mitochondria of dopamine neurons where it interacted with the inner membrane, interfered with complex I activity and increased the production of ROS (Devi et al. 2008; Luth et al. 2014). Oligomeric and post-translationally modified species of α-syn have also been shown to inhibit mitochondrial protein import leading to reduced mitochondrial respiration and enhanced production of ROS (Di Maio et al. 2016).

Accumulation of misfolded proteins can trigger ER stress which leads to UPR activation. ER stress and UPR are also common features of the PD pathogenesis and closely associated with aggregated α-syn (Mercado et al. 2013). Neuropathological analysis of *postmortem* brain tissue derived from PD patients revealed increased immunoreactivity of the UPR markers phosphorylated PERK, IRE1α and eukaryotic initiation factor 2α (eIF2α) in dopamine neurons of the SNpc (Heman-Ackah et al. 2017; Hoozemans et al. 2007; Mercado et al. 2018). Phosphorylated PERK and IRE1α also colocalized with α-syn inclusions. In line, genetic and neurotoxin rodent models of PD as well as induced pluripotent stem cell -derived neurons overexpressing α-syn have shown similar ER stress phenotype and activated UPR. Of note, ER-resident CDNF and MANF serve as potential therapeutic agents to mitigate ER stress in PD.

2.3.2.7 Selective vulnerability of the SNpc dopamine neurons

The particular susceptibility of the dopamine neurons in the SNpc to the neuronal damage is not fully understood. As described earlier, nigral dopamine neurons are autonomous pacemakers spiking at low frequencies (2-5 Hz) and exhibiting broad action potentials (>2 ms). Unlike most of the other pacemaking neurons that rely on Na^+ channels, the SNpc dopamine neurons have a strong reliance on L-type voltage-dependent Ca^{2+} channels with a $Ca_v1.3$ subunit leading to increased Ca^{2+} entry into the cells (Surmeier and Schumacker 2013). In addition, the SNpc dopamine neurons have low intrinsic Ca^{2+} -buffering capacity. In comparison, the dopamine neurons in the VTA, which are also slow pacemakers but less prone to degeneration in PD, have much lower L-type $Ca_v1.3$ Ca^{2+} channel density, do not manifest significant intracellular Ca^{2+} oscillations and express high levels of the Ca^{2+} -buffering protein calbindin. The maintenance of Ca^{2+} gradient across the plasma membrane is energetically expensive. Because of the high energy demand, nigral dopamine neurons are strongly dependent on the mitochondrial ATP production. Increased mitochondrial oxidative phosphorylation gives rise to high levels of ROS generation and oxidative stress in the neurons. High metabolic demands also increase the risk of bioenergetic crisis in case of an episodic disruption in the mitochondrial function. Reduced Ca^{2+} influx might explain the potentially reduced risk of PD associated with the use of calcium channel blockers (Noyce et al. 2012).

The SNpc dopamine neurons have a massively arborized axonal tree with a very high number of axon terminals which causes a great bioenergetic burden on these cells (Giguère et al. 2018). Elevated energetic requirements can deplete cellular antioxidant storages and subject neurons to chronic oxidative stress. It is estimated that up to half of the energy consumed by the SNpc dopamine neurons is used to action potential firing and neurotransmitter release. Thus, stressed neurons may try to adapt to excessive metabolic needs by dysregulating axon terminals as seen in the early phases of PD.

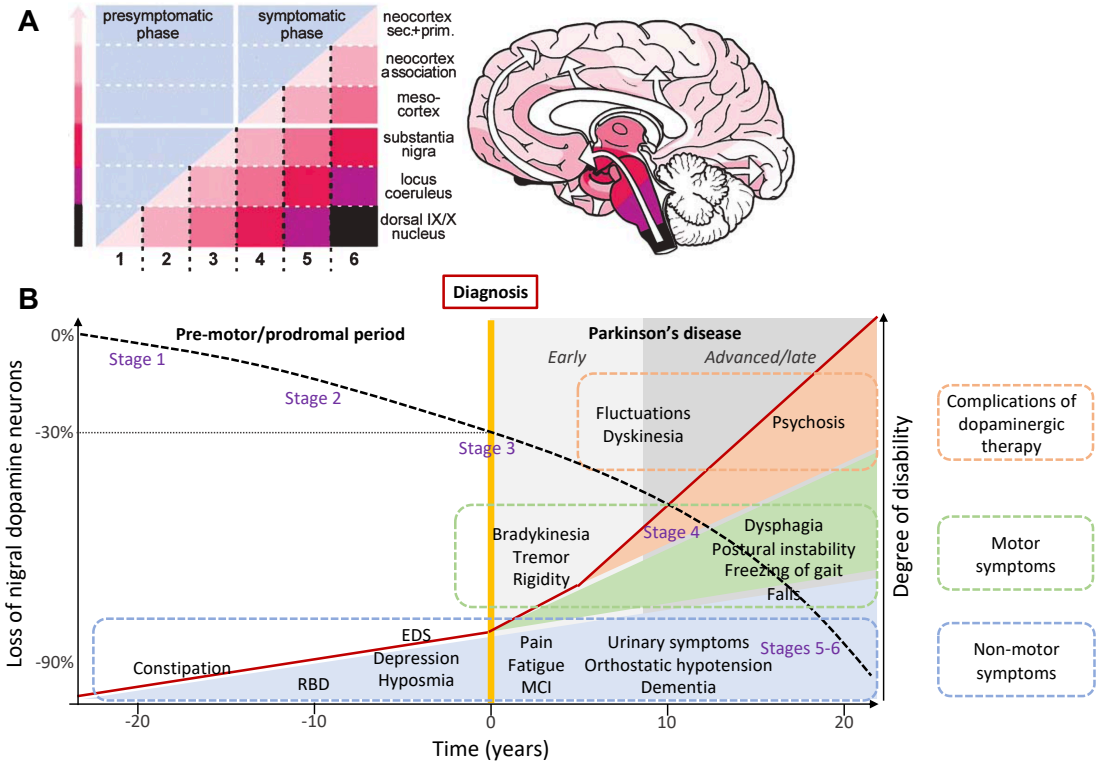

Figure 2.10. (A) Braak staging system of Lewy pathology spreading. In stage 1, Lewy inclusions start to appear in the dorsal motor nucleus of the vagus nerve in the medulla oblongata and olfactory system. In stage 2, locus coeruleus and caudal raphe nuclei in the pontine tegmentum become affected. In stage 3, the pathology ascends to the midbrain including substantia nigra, amygdala and the cholinergic nuclei of the basal forebrain. In stage 4, the pathology reaches thalamus, hippocampus and temporal mesocortex. In stages 5 and 6, several cortical areas, including prefrontal, primary sensory and motor cortices, and finally, the entire neocortex become involved. Growing severity of the Lewy pathology is shown by increasing shading (red, violet, black). Reproduced with permission from Springer Nature: Journal of Neural Transmission, Braak et al. (2003b), © Springer Nature 2003 **(B) Time course of Parkinson's disease progression**. Accumulating clinical symptoms (red line) are associated with the loss of nigral dopamine neurons (dashed black line) and Braak stages of Lewy pathology spreading (in violet). Up to two decades long pre-motor/prodromal phase (stages 1 and 2) characterized by specific, mainly autonomic, olfactory and sleep-related non-motor symptoms can precede the diagnosis (time 0 years). During stage 3, the characteristic motor features start to manifest, leading to the diagnosis between stages 3 and 4. Additional non-motor symptoms develop with the disease progression, causing significant disability. Stages 5 and 6 are associated with advanced disease with debilitating axial motor symptoms. Complications of dopaminergic therapy contribute to clinical disability. EDS - excessive daytime sleepiness; MCI - mild cognitive impairment; RBD - REM sleep behavior disorder. Adapted with permission from Elsevier: The Lancet, Kalia and Lang (2015), © Elsevier 2015.

Another hypothesis linking increased oxidative stress to the vulnerability of the SNpc neurons is that oxidation of cytosolic dopamine and its metabolites leads to the excessive production of cytotoxic free radicals, dopamine-quinones, which may disrupt the function of e.g. mitochondrial protein complexes or DAT (Greenamyre and Hastings 2004; Giguère et al. 2018). This may well be a contributing factor in the pathogenesis of PD but probably not the principal culprit. Dysfunctional iron homeostasis causing overload of free iron in the SNpc has also been proposed to trigger oxidative stress in PD. However, this hypothesis has remained controversial thus far (Sian-Hülsmann et al. 2011).

2.3.3 Symptoms and diagnosis of Parkinson's disease

The characteristic motor manifestations of PD include bradykinesia, rigidity, resting tremor, postural instability and gait disturbances (Kalia and Lang 2015). Bradykinesia is the precondition for the clinical diagnosis of PD [UK Parkinson's Disease Society Brain Bank clinical diagnostic criteria (Hughes et al. 1992); revised by Movement Disorder Society Clinical Diagnostic Criteria for Parkinson's disease (Postuma et al. 2015)]. In addition, at least one other motor feature is required for the diagnosis: muscular rigidity, 4-6 Hz resting tremor, and/or postural instability. The diagnosis is confirmed by excluding possible alternative disorders and with the presence of at least three supportive criteria such as unilateral onset, progressive symptoms, marked responsiveness to L-DOPA treatment, L-DOPA-induced dyskinesias and clinical course of 10 years or more. The diagnosis is based on clinical neurological examination but neuroimaging techniques such as single-photon emission computed tomography (SPECT), magnetic resonance imaging (MRI) and PET can be used in differential diagnosis and tracking the progression of the disease (see below chapter 2.3.4) (Politis 2014). Neuropathological overlap and heterogeneity in clinical manifestations complicate the differential diagnosis between early-stage PD and atypical parkinsonian syndromes such as multiple system atrophy (MSA), progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD). Despite advanced diagnostic tools, full certainty of the diagnosis cannot be achieved *pre mortem*; 75-95% of the diagnoses are confirmed by histological identification of Lewy pathology upon autopsy (Postuma et al. 2015).

Apart from the motor impairment, PD patients suffer from heterogenous non-motor symptoms such as olfactory deficits, cognitive impairment, psychiatric symptoms, sleep disturbances, autonomic dysfunctions, pain and fatigue (Khoo et al. 2013; Kalia and Lang 2015) (Figure 2.10.B). Some of these symptoms are of dopaminergic origin while the others are non-dopaminergic, reflecting the multisystem nature of PD (Fox et al. 2008; Chaudhuri and Schapira 2009). Oftentimes, it is the non-motor symptoms, rather than the motor symptoms, that dominate the decline of patients' health-related quality of life (Barone et al. 2009; Martinez-Martin et al. 2011; Duncan et al. 2014). Cognitive decline is one of the most prevalent and debilitating non-motor feature of PD (Aarsland et al. 2009; O'Callaghan and Lewis 2017). Mild cognitive impairment (MCI) is considered as an intermediate state that typically progresses to PD dementia. In some patients, MCI is recognized already at the earliest stages of the disease. The likelihood of manifesting MCI or dementia considerably grows as the disease progresses. On the whole, the burden of non-motor symptoms increases over time. Psychiatric symptoms such as anxiety, depression and

psychosis, and autonomic dysfunctions including constipation, dysphagia, urinary incontinence and postural hypotension are common features in the later stages of PD and resistant to L-DOPA treatment. These late-stage symptoms are predictive for institutionalization and, eventually, mortality.

Certain non-motor symptoms can precede the onset of the motor impairment and clinical diagnosis by more than a decade (Postuma et al. 2012) (Figure 2.10.B). These prodromal, or pre-motor, symptoms typically include impaired olfaction (hyposmia), constipation, orthostatic hypotension, rapid eye movement (REM) sleep behavior disorder (RBD), depression and excessive daytime sleepiness. Efforts have been made to identify prodromal symptoms that could be used as diagnostic markers to screen for PD before the motor manifestations. Depression, constipation and idiopathic RBD are currently the strongest candidates that, together with family history of PD, could support the diagnosis but the specificity and predictive value of these markers are still insufficient (Howell and Schenck 2015; Noyce et al. 2012).

2.3.4 Biomarkers of Parkinson's disease

Biomarkers can be used to assist in early and differential diagnosis and predict the course of a disease. They can also help to assess response to a therapeutic intervention. An optimal biomarker is easily accessible, inexpensive, sensitive and reproducible (Yilmaz et al. 2019). Currently, there are several biomarker candidates available for PD but none of them is specific or reliable enough to be used alone to diagnose or follow the progression of the disease (Kalia 2018; Parnetti et al. 2019). This shortage mainly arises from the heterogeneity of PD on genetical, pathophysiological and clinical level. The potential approaches to identify new biomarkers include biomolecular, histological and "omics" based analyses of biofluids and peripheral tissue samples (biochemical biomarkers), brain imaging modalities (imaging biomarkers), electrophysiological and digital biomarkers (other biomarker modalities). In general, the diagnostic utility of these approaches remains to be validated in large trials with standardized study protocols before their introduction to clinical practice. The use of a combination of biomarkers could detect multiple pathological aspects and result in improved diagnostic accuracy.

2.3.4.1 Biochemical biomarkers

Identifying biomarkers from body fluids that are easily accessible (e.g. blood, saliva or urine) would be desirable, but due to the peripheral nature of these matrixes, the sensitivity and specificity issues might limit their utility (Chahine and Stern 2017). Accumulating evidence suggests diagnostic value for cerebrospinal fluid (CSF) and blood biomarkers reflecting the pathophysiology of PD such as α-syn species, lysosomal enzymes, neurofilaments and markers of Aβ and tau pathology (Parnetti et al. 2019). Especially various forms of α-syn, total monomeric, phosphorylated, oligomeric and aggregated species, have been investigated intensively (Atik et al. 2016; Visanji et al. 2017). The close contact of the CSF to the brain makes it a potential source for diagnostic markers reflecting the ongoing pathological processes and metabolic changes in the CNS (Constantinescu and Mondello 2013; Eusebi et al. 2017; Parnetti et al. 2019). It is not, however, an ideal matrix for longitudinal monitoring because of the need for repeated lumbar

punctures and risk for blood contamination which may disturb some analyses. Nevertheless, the CSF levels of α-syn species have provided encouraging results as biomarker candidates for PD. The total concentration of α-syn in the CSF seems to be lower, whereas the levels of α-syn oligomers and phosphorylated α-syn are elevated in PD patients as compared to healthy controls.

Efforts have also been made to quantify plasma levels of α-syn species, neurofilaments and inflammatory cytokines as potential biomarkers for PD. Plasma sampling would provide a convenient route for early diagnosis and longitudinal disease monitoring. However, the measurement of total α-syn from plasma has provided inconsistent outcomes, partly because plasma samples are easily contaminated with α-syn containing erythrocytes (Constantinescu and Mondello 2013; Parnetti et al. 2019). Quantification of α-syn oligomers and phosphorylated α-syn from plasma provides an alternative approach which has shown more concordant results with increased levels in PD patients.

Untargeted "omics" techniques in combination with bioinformatic tools provide a powerful approach to detect large amounts of analytes in various biosamples and then compare these analytical "fingerprints" between patients with healthy controls. Metabolomics has been utilized to detect PD-related alterations for example in plasma and CSF levels of amino acids, fatty acids and sugars (LeWitt et al. 2013; Trupp et al. 2014; Willkommen et al. 2018). Also, attempts to use proteomics and transcriptomics data collected from the body fluid samples have been made (Scherzer et al. 2007; Halbgebauer et al. 2016; Santiago et al. 2018). These "omics" based biomarkers have the potential to unravel new aspects of PD pathogenesis and aid in the future diagnostics of the disease.

In consistence with the evidence suggesting that α-syn pathology appears in the PNS before propagating to the brain, attention has been paid to histological examination of phosphorylated α-syn deposits in various peripheral tissue biopsies from PD patients (Cersosimo and Benarroch 2012; Lee et al. 2017). For example, submandibular gland biopsy and subsequent detection of phosphorylated α-syn aggregates in the autonomic nerve fibers have shown promise as an early histological biomarker (Adler et al. 2016; Vilas et al. 2016). Histological analysis of phosphorylated α-syn in dermal nerve fibers in skin biopsies is also a sensitive candidate for early diagnosis (Doppler et al. 2016).

2.3.4.2 Imaging biomarkers

Brain imaging techniques are widely studied tools that can provide support for early, differential and prognostic diagnosis. Multimodal imaging allows the visualization of structural and functional changes in the brain and can reveal pathophysiological mechanisms underlying the disease processes (Saeed et al. 2017). As imaging modalities are non-invasive, they are well suited for longitudinal tracking of disease progression and therapeutic responses.

Structural MRI is used to assess regional tissue atrophy and serves as a potential aid for PD diagnosis (De Marzi et la. 2016; Frosini et al. 2017; Pyatigorskaya et al. 2017). Neuromelanin-sensitive MRI imaging of the SNpc and locus coeruleus has shown promising diagnostic accuracy

in differentiating PD patients from healthy controls (Castellanos et al. 2015). Functional MRI (fMRI) can be used to detect changes in brain network activities by measuring cerebral oxygen-rich blood flow. Significantly reduced functional connectivity in resting-state fMRI have been reported within the basal ganglia of patients with early-stage PD (Rolinski et al. 2015; Szewczyk-Krolikowski et al. 2014). Indeed, resting-state fMRI of basal ganglia connectivity is a promising diagnostic tool that may help to identify patients at risk of developing PD (Rolinski et al. 2016).

SPECT is a useful technique to assess the integrity of the nigrostriatal dopamine pathway (Saeed et al. 2017). Gamma-emitting cocaine analogues, such as ^{123}I-FP-CIT and ^{123}I-β-CIT, bind selectively to presynaptic DAT and can be used to measure the density of nigrostriatal fibers. Reduced striatal uptake in DAT-SPECT is a characteristic readout in PD patients when compared to healthy controls. A meta-analysis suggests that DAT-SPECT is useful in the diagnosis of early PD and in differentiating PD from essential tremor and vascular parkinsonism (Vlaar et al. 2007). DAT-SPECT can also be utilized to follow disease progression because striatal binding seems to correlate to disease severity stages (Benamer et al. 2000; Brücke et al. 1997).

PET is another imaging modality taking advantage of radioactive ligands. It can be utilized to gauge the integrity of dopaminergic system, cerebral glucose metabolism, pathological Aβ and tau protein accumulation and neuroinflammation (Saeed et al. 2017). 6-^{18}F-fluoro-L-dopa (^{18}F-DOPA) is a PET tracer that is converted to ^{18}F-dopamine by AADC. Thus, ^{18}F-DOPA measures the activity of AADC and provides an indirect estimation of the presynaptic dopamine storage pools. In PD, striatal ^{18}F-DOPA uptake is decreased and associated with the progression of the disease. PET imaging of presynaptic DAT, using for example ^{18}F-FP-CIT or ^{11}C-methylphenidate, shows reduced striatal uptake in PD patients, in line with DAT-SPECT findings. However, compensatory mechanisms, such as upregulation of AADC activity and downregulation of DAT, in response to neurodegeneration may inflict biases in AADC-PET, DAT-PET or DAT-SPECT -based estimates of the nigrostriatal injury (Lee et al. 2000). VMAT2 imaging, instead, for example with ^{11}C-dihydrotetrabenazine (^{11}C-DTBZ) PET, seems to be less prone to the compensatory changes, thus producing more reliable readouts of the nigrostriatal degeneration in PD. Despite considerable efforts to develop an α-syn-specific radiotracer, a direct method for α-syn imaging *in vivo* is still lacking (Eberling et al. 2013). Such a tracer would allow for tracking α-syn pathology spreading over time and monitoring the efficacy of α-syn targeting therapies.

2.3.4.3 Other biomarker modalities

Electrophysiological techniques, such as electroencephalography (EEG), can be used to identify candidate biomarkers for PD. For example, lower background rhythm frequency and increased relative power of delta and theta oscillations in resting-state EEG show promise as predictive biomarkers for cognitive deterioration in PD (Caviness et al. 2015; Klassen et al. 2011). In a prospective EEG study, slower background frequency during REM sleep together with increased delta and theta band powers predicted the later development of PD dementia (Latreille et al. 2016).

With recent technological advancements, digital biomarkers form a rapidly emerging field of research to improve the longitudinal tracking of neurodegenerative diseases (Espay et al. 2016; Hansen et al. 2018; Baker et al. 2019; Kourtis et al. 2019; Mahadevan et al. 2020). Digital biomarkers refer to the use of inbuilt sensors in portable (e.g. smartphone), wearable (e.g. smart watch) or implantable devices allowing for continuous active and/or passive data collection of biological (e.g. blood glucose), physiological (e.g. heart rate or blood pressure) or functional (e.g. motor activities, speech or facial expression) parameters (Dorsey et al. 2017).

2.3.5 Current treatments for Parkinson's disease

Today, there is no cure available for PD patients. The current therapies ameliorate the disease associated motor symptoms by replenishing striatal dopamine deficiency with the BBB-penetrating dopamine precursor L-DOPA or boosting dopaminergic transmission with dopamine receptor agonists or drugs that prevent the degradation of dopamine (Armstrong and Okun 2020; Fox et al. 2018). The efficacy of these dopamine-based therapies, however, gradually diminishes as the disease progresses and they are unable to delay or reverse the neurodegenerative processes underlying the disease. Thus, there is an unmet medical need for a disease-modifying therapy for PD. The currently used treatments for motor and non-motor symptoms of PD are presented in Table 2.2.

L-DOPA has been the cornerstone of PD treatment since the seminal experiments in 1961 by Walther Birkmayer and Oleh Hornykiewicz who showed the striking effect of the first intravenous injections of L-DOPA on akinetic PD patients (Birkmayer and Hornykiewicz 1961; Hornykiewicz 2002). L-DOPA, given in combination with a peripheral AADC inhibitor, has remained the "the gold standard" of PD treatment until today due to its efficacy, tolerability and low cost (Lewitt 2015).

Although L-DOPA has remarkable therapeutic efficacy in the early-stage PD, the progressive loss dopamine neurons results in narrowing therapeutic window (Armstrong and Okun 2020; Chou et al. 2018; Connolly and Lang 2014). The required dose escalations, the short half-life of L-DOPA and its intermittent availability after oral administration cause fluctuations in drug levels in the brain. Eventually, the effect of a L-DOPA dose starts to wear off faster causing the re-emerge of parkinsonian motor and non-motor symptoms ("off" state) while the highest L-DOPA concentrations cause troublesome dyskinesias, i.e. involuntary, nonrhythmic chorea-type movements.

The motor fluctuations between the "off" state and the "on" state, when the symptoms are controlled but often associated with peak-dose dyskinesias, are proposed to result from the reduced capacity of the degenerating synaptic terminals to store extra dopamine and alterations in the striatal postsynaptic signaling cascades (Chou et al. 2018; Marsden and Parkes 1976). The pathogenic mechanisms of dyskinesias remain to be fully elucidated but appear to include pulsatile stimulation of dopamine receptors, conversion of L-DOPA to dopamine in serotonin neurons and subsequent unphysiological release of dopamine from the striatal serotonergic terminals as a "false neurotransmitter", corticostriatal glutamatergic overdrive, stimulation of

nAChRs on dopaminergic terminals and aberrant activation of direct-pathway MSNs (Chase and Oh 2000; Espay et al. 2018).

Over time, the motor fluctuations become increasingly difficult to manage and their prevalence increases (Armstrong and Okun 2020; Chou et al. 2018; Connolly and Lang 2014). A common strategy to reduce them is to divide L-DOPA dosage into smaller but more frequent doses. Various combinations of medications are also commonly used together with L-DOPA to increase therapeutic benefit while limiting the high dose-related side-effects. The most common adjunct medications are dopamine receptor agonists and MAO-B and COMT inhibitors (Fox et al. 2018). Subcutaneous apomorphine (D1 and D2 agonist) injections, self-administered via an injection pen, can be used to achieve faster medication response for those who suffer from severe “off” periods.

The abovementioned strategies are effective to a certain extent, but as the disease advances, some patients become irresponsive to conventional medication adjustments. In these cases, device-aided interventions such as intracranial deep brain stimulation (DBS), small dose infusion of L-DOPA-carbidopa gel into jejunum or continuous subcutaneous apomorphine infusion may be considered to treat difficult motor fluctuations and dyskinesias (Armstrong and Okun 2020; Fox et al. 2018; Martinez-Martin et al. 2015; Volkmann et al. 2013). The selection between these advanced therapy options is made based on case-by-case evaluation.

In addition to the pharmacological interventions, physical exercise regimens, physiotherapy and occupational therapy may be useful in improving motor performance or delaying the disease progression (Armstrong and Okun 2020; Fox et al. 2018; Mak et al. 2017). Strength and aerobic endurance training programs appear to produce long-lasting positive effects on the physical functioning of PD patients. Balance, gait, Tai chi and dance exercises have also been shown to reduce falls and improve mobility and walking capacity. The physical exercise interventions should be sustained, intensive, multimodal and start at an early stage of the disease in order to produce maximal benefits. Additionally, speech therapy seems to be a useful strategy for managing hypophonia and dysphagia that are common symptoms in PD.

The non-motor symptoms substantially increase the clinical and financial burden of PD but remain largely refractory to the existing dopaminergic drugs (Armstrong and Okun 2020; Connolly and Lang 2014; Seppi et al. 2019). The management of non-motor symptoms is symptomatic and extrapolated from the general treatment guidelines for these symptoms in non-PD individuals with certain disease-specific elaborations. Some symptoms, such as MCI, have remained without evidence-based treatment options.

Table 2.2. Currently used treatments for motor and non-motor symptoms of Parkinson's disease. References provided in the main text

	Category	Interventions	Mechanism or mode of action	Indications	Common adverse effects
Treatments for motor symptoms	L-DOPA preparations	L-DOPA-carbidopa/ benserazide, *p.o.* immediate release	L-DOPA: Precursor of DA, crosses the BBB → AADC converts to DA in the CNS → increases DA content in the remaining DA neurons	Early symptomatic monotherapy, motor fluctuations	Nausea, vomiting, diarrhea, loss of appetite, orthostatic hypotension, dizziness, cardiac arrhythmia, dyspnea, sleepiness, insomnia, restlessness, depression, confusion, hallucinations, strange dreams, motor fluctuations, peak-dose dyskinesias
		L-DOPA-carbidopa/ benserazide, *p.o.* extended release	Carbidopa/ benserazide: peripheral AADC inhibitor → prevents the conversion of L-DOPA to DA outside the brain → increases L-DOPA concentration in the CNS and reduces peripheral side-effects of DA		
		L-DOPA-carbidopa intestinal gel infusion	See above, reduces fluctuations in the plasma concentration of L-DOPA	Advanced motor fluctuations and dyskinesias that are irresponsive to conventional medication adjustments	See above + weight loss, constipation, falls, anxiety, neuropathy + complications of surgical device insertion, abdominal pain, irritation or infection at the stoma, tube dislocation or obstruction
	Non-ergot DA agonists	Pramipexole	Activates postsynaptic D2-like DA receptors	Early symptomatic monotherapy, L-DOPA adjunct, motor fluctuations, depression	Nausea, vomiting, constipation, sleepiness, orthostatic hypotension, dizziness, edema, hallucinations, impulsive-compulsive disorders*
		Ropinirole		Like Pramipexole - depression	
		Rotigotine (transdermal patch)			
		Apomorphine, intermittent *s.c.* injections	Activates postsynaptic D2- and D1-like DA receptors	Motor fluctuations	The same as other non-ergot DA agonists. In addition, yawning, runny nose, heart palpitation, dyskinesias, priapism, injection/ infusion site reactions and nodules
		Apomorphine, continuous *s.c.* infusion		Advanced motor fluctuations that are irresponsive to conventional medication adjustments	
	MAO-B inhibitors	Selegiline	Blocks the degradation of DA to DOPAL in the CNS → increases DA concentration in the CNS	Early symptomatic monotherapy, L-DOPA adjunct, motor fluctuations	Headache, exacerbation of L-DOPA adverse effects
		Safinamide			
		Rasagiline			Headache, arthralgia, dyspepsia, constipation, flu-like syndrome, exacerbation of L-DOPA adverse effects
	COMT inhibitors	Entacapone	Blocks the degradation of L-DOPA to 3-OMD in periphery → increases L-DOPA concentration in the CNS	Motor fluctuations	Dark-colored urine, GI complications, dry mouth, increased sweating, angina pectoris, exacerbation of L-DOPA adverse effects
		Opicapone			Like Entacapone + muscle spasms + increased plasma levels of creatine kinase
		Tolcapone	Blocks the degradation of L-DOPA to 3-OMD in periphery as well as L-DOPA and DA degradation to 3-OMD and 3-MT in the CNS, respectively → increases L-DOPA and DA concentration in the CNS		Like Entacapone + hepatotoxicity
	Anticholinergics[a]	Biperiden	Centrally active muscarinic receptor (especially M1) antagonist → reduces the relative cholinergic overactivity in basal ganglia	Early symptomatic monotherapy, L-DOPA adjunct, tremor-prominent symptoms, drooling, neurogenic bladder dysfunction	Anticholinergic side-effects**, nausea, dizziness, anxiety, hallucinations
		Trihexyphenidyl			
	NMDA receptor antagonist	Amantadine	Not completely understood. Blocks NMDA and nAChRs → decreases corticostriatal glutamatergic activity; increases DA and NA release and blocks reuptake of DA	Early symptomatic monotherapy[b], L-DOPA adjunct[b], dyskinesias	Orthostatic hypotension, nausea, dry mouth, blurred vision constipation, urinary retention, edema, insomnia, confusion, hallucinations, livedo reticularis
	Atypical antipsychotic	Clozapine	5-HT_2 receptor favoring antagonist with lower affinity for D1 and D2 receptors	Dyskinesias	Sleepiness, dizziness, sedation, tachycardia, constipation, orthostatic hypotension, drooling, risk of agranulocytosis → regular monitoring of the blood count

	Functional neurosurgery	Bilateral STN DBS[a]	Not completely understood. High frequency electric stimulation via implanted electrodes causes global inhibition of the target nucleus → mitigates abnormal oscillations in basal ganglia	Advanced motor fluctuations and dyskinesias that are irresponsive to conventional medication adjustments	Dysarthria, impaired working memory, falls, surgery related complications, infections, device related problems, depression
		Bilateral GPi DBS[a]			Like Bilateral STN DBS - depression
		Unilateral VIM DBS[a] or thalamotomy	Mitigates abnormal oscillations in the thalamus	Medication-refractory tremor as the main symptom	Like Bilateral STN DBS
Treatments for non-motor symptoms	Cholinesterase inhibitors	Rivastigmine	Decrease the hydrolysis of ACh → increases ACh concentration at cholinergic synapses in the brain	Dementia	GI complications, weight loss, bradycardia, vivid dreams, risk for exacerbation of rest tremor
		Donepezil			
		Galantamine			
	Antidepressants	Fluoxetine, paroxetine, citalopram, sertraline	SSRI	Depression	Nausea, anorexia, drowsiness, sexual dysfunction, akathisia
		Venlafaxine	SNRI		Drowsiness, insomnia, sexual dysfunction, gastrointestinal symptoms
		Nortriptyline, desipramine, amitriptyline	Tricyclic antidepressants		Anticholinergic side-effects**, orthostatic hypotension, ventricular arrhythmias, heart block, sleepiness, sexual dysfunction, weight gain
	Atypical antipsychotics	Clozapine	5-HT_2 receptor favoring antagonists with lower affinity for D1 and D2 receptors (First, potentially hazardous medications such as anticholinergics, amantadine, DA agonists, MAO-B and COMT inhibitors are reduced)	Psychosis, hallucinations	See above
		Quetiapine			Extrapyramidal symptoms, sedation
		Pimavanserin			Nausea, constipation, edema, confusion, weight gain
	Hypnotic	Eszopiclone	Positive allosteric modulator of $GABA_A$ receptor → increases Cl^- influx into the neurons	Insomnia	Headache, dry mouth, nausea, and dizziness
	Hormone	Melatonin	Melatonin receptor agonist → regulates physiological sleep-wake cycle	Insomnia, RBD	Not common, rarely daytime sleepiness, dizziness, headache
	Tranquilizer	Clonazepam[a]	Positive allosteric modulator of $GABA_A$ receptor → increases Cl^- influx into the neurons	RBD	Sedation, drowsiness confusion
	Mineralocorticoid	Fludrocortisone	Increases the reabsorption of Na^+ and water in the kidney → increases blood volume and blood pressure	Orthostatic hypotension	Hypertension, metabolic abnormalities, GI complications, myopathy
	Sympathomimetics	Midodrine	Activates adrenergic α_1-receptors in the vasculature → increases vascular tone and blood pressure		Hypertension, nausea, weakness, heartburn, headache, chills
		Droxidopa	Precursor of NA, AADC converts to NA in the PNS → maintains blood pressure and blood flow		Hypertension, tachycardia, nausea, vomiting, headache
	Osmotic laxative	Macrogol	Inert substance that osmotically retains water in the bowel → softens and increases the volume of the stool	Constipation	Electrolyte imbalance, increased bowel gas, abdominal pain, nausea, diarrhea
	Dietary supplements	Probiotics and prebiotic fiber	Stimulate the growth and activity of beneficial micro-organisms in the gut → aid digestion		Bloating, increased bowel gas, diarrhea
	Prostaglandin E1 derivative	Lubiprostone	Activates Cl^--channels on the apical surface of GI epithelial cells → increases fluid secretion, softens the stool and promotes bowel motility		Nausea, diarrhea, headache, bloating, abdominal pain, flatulence
	Antidiuretic hormone analog	Desmopressin	Increases the presence of aquaporin channels in the distal nephron by binding to V2 receptors → increases water reabsorption from urine → decreases the total amount of urine	Neurogenic bladder dysfunction	Hyponatremia, hypertension, edema, urinary tract complications, headache, dizziness, GI complications, sleepiness
	Neurotoxin	Botulinum toxin injection into salivary glands	Prevents the release of ACh from the postganglionic parasympathetic axon terminals → reduction of saliva production	Drooling	Dysphagia, dry mouth, injection-related discomfort

3-OMD, *3-O-methyldopa*; ACh, *acetylcholine*; DA, *dopamine*; GI, *gastrointestinal*; NA, *noradrenaline*; nAChR, *nicotinic acetylcholine receptor*; *p.o., peroral*; *s.c., subcutaneous*; SNRI, *serotonin and noradrenaline reuptake inhibitor*; SSRI, *selective serotonin reuptake inhibitor*; VIM, *ventralis intermedius nucleus of the thalamus*; see text for other abbreviations.

* Impulsive-compulsive disorders include pathological gambling, hypersexuality, binge eating, compulsive shopping and overuse of dopaminergic medications.

** Anticholinergic side-effects include cognitive impairment, confusion, tachycardia, vertigo, impaired accommodation, increased intraocular pressure, dry mouth, urinary retention and constipation.

[a] For cognitively intact patients; [b] Not commonly used anymore

2.4 Animal models of Parkinson's disease

In spite of fast developments in *in vitro* disease models, organoids and computer-aided drug development tools, none of these techniques have been able to completely replace the pre-clinical studies in animal models. *In vivo* experiments still form a crucial step where the efficacy and safety of a novel therapy can be tested in a complex, whole-organism setting before entering into the first human trials. Furthermore, animal models provide a powerful means to gain deeper understanding of pathogenic mechanisms which, in turn, contribute to establishing novel targets for disease-modifying therapies. The 3R principles lay the groundwork for performing responsible and humane animal research: they aim at replacing, reducing and refining animal testing whenever possible.

Reflecting the complex nature of the disease, a wide array of strategies and organisms has been used to produce *in vivo* models of PD (Dauer and Przedborski 2003; Gubellini and Kachidian 2015). They all model certain aspects of the disease, but none of the current models can fully recapitulate the neuropathology and symptoms of PD. Although non-mammals provide simple and powerful systems to model the pathobiology of PD, the focus here is on the most widely used rodent and NHP models. They can be classified into four main categories: pharmacological, neurotoxin-based, genetic and α-syn pathology spreading models. The main features of these models are summarized in Figure 2.11.

2.4.1 Pharmacological models

Reserpine and alpha-methyl-*p*-tyrosine (AMPT) are pharmacological agents that have been used to induce parkinsonism in rodents and NHPs (Carlsson et al. 1957; Windle and Cammermeyer 1958; Bezard and Przedborski 2011). They deplete brain dopamine and other catecholamines by inhibiting VMAT2 (reserpine) or TH (AMPT) while leaving dopamine neurons intact. Striatal dopamine depletion leads to PD-like motor deficits.

2.4.2 Neurotoxin-based models

Neurotoxins produce a prominent and rapid degeneration of the nigrostriatal dopamine system resulting in a clear motor impairment in experimental animals. 6-OHDA is a widely used catecholaminergic neurotoxin (Simola et al. 2007). 6-OHDA is a hydrophilic structural analogue of catecholamines that is unable to cross the BBB. Thus, it needs to be directly injected into the target brain structure in a stereotaxic surgery. Prophylactic peripheral administration of a noradrenaline transporter blocker, such as desipramine, is common to prevent the uptake of 6-OHDA into noradrenergic nerve terminals.

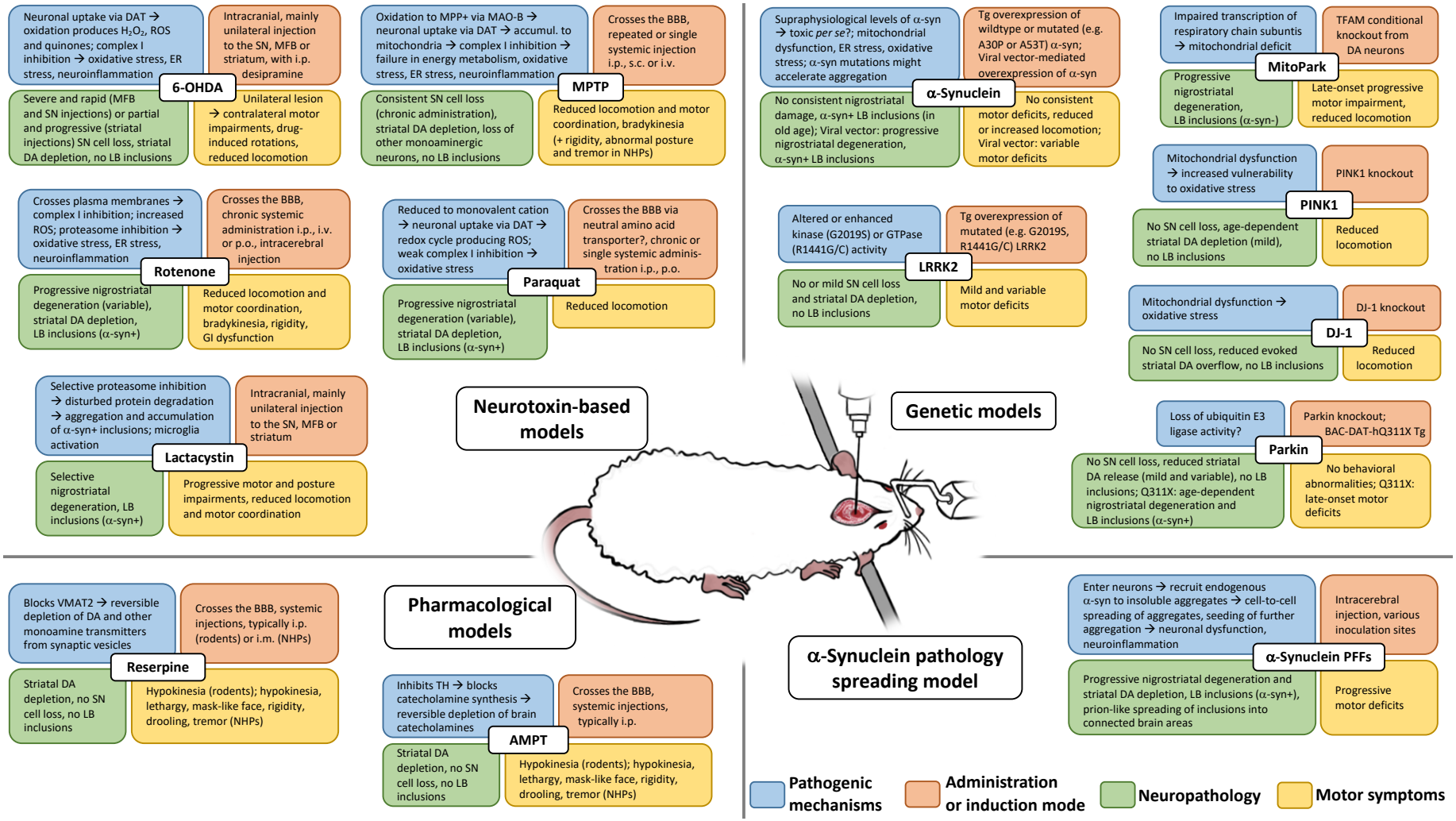

Figure 2.11. Animal models of Parkinson's disease. The main characteristics of pharmacological, neurotoxin-based, genetic and alpha-synuclein pathology spreading models of PD. AMPT - Alpha-methyl-*p*-tyrosine; α-syn+/- - alpha-synuclein positive/negative; BAC - bacterial artificial chromosome; BBB - blood-brain barrier; DA - dopamine; DAT - dopamine transporter; ER - endoplasmic reticulum; LB - Lewy body-like; MAO-B - monoamine oxidase B; MFB - medial forebrain bundle; NHP - non-human primate; PFF - α-syn preformed fibrils; ROS - reactive oxygen species; SN - substantia nigra; TFAM - mitochondrial transcription factor A; Tg - transgenic, TH - tyrosine hydroxylase; VMAT2 - vesicular monoamine transporter 2. References are provided in the text. Figure drawn by the author and Fanni-Sofia Renko.

Urban Ungerstedt developed a widely used unilateral 6-OHDA lesion model of PD (Ungerstedt 1968; Ungerstedt and Arbuthnott 1970; Ungerstedt 1971). Unilateral injection of 6-OHDA into the SNpc, medial forebrain bundle (MFB) or dorsal striatum induces degeneration of nigrostriatal dopamine neurons on the injected side accompanied with contralateral motor impairment (Perese et al. 1989; Sauer and Oertel 1994; Kirik et al.1998; Deumens et al. 2002). In severe lesion models (SNpc and MFB lesions), marked dopamine depletion in the striatum leads to supersensitization of postsynaptic dopamine receptors. Unilateral lesions produce asymmetrical motor deficits that can be easily assessed with behavioral tests. For example, amphetamine-induced dopamine release in the intact striatum elicits ipsilateral rotational behavior, whereas activation of supersensitized dopamine receptors in the lesioned striatum by apomorphine induces turning to the contralateral direction. Important advantages of unilateral lesion model include increased survival of the experimental animals as compared to bilateral lesions and the possibility to use contralateral hemisphere as an internal control in the analyses.

MPTP is another dopaminergic neurotoxin most commonly used to model PD in mice and NHPs, but not in rats which were found to be resistant to the toxin (Bezard and Przedborski 2011; Dauer and Przedborski 2003). Lipophilic MPTP readily crosses the BBB, and thus is suitable for systemic administration. In the brain, MAO-B converts MPTP to the toxic metabolite MPP+. Utilizing mitochondrial transmembrane potential MPP+ concentrates into the mitochondria where it inhibits the complex I of the electron transport chain (Chiba et al. 1984; Nicklas et al. 1985). MPTP intoxication also causes ER stress, neuroinflammation and upregulation of pro-apoptotic Bax and downregulation of anti-apoptotic Bcl-2 in dopamine neurons (Vila et al. 2001; Mercado et al. 2013; Lee et al. 2019). In mice, strain, gender, age and body weight affect the sensitivity to MPTP (Jackson-Lewis and Przedborski 2007). Prolonged subcutaneous or intraperitoneal administration of low or moderate doses of MPTP to C57/Bl6 mice produces a robust PD-like phenotype with defined apoptosis of the SNpc dopamine neurons and impaired motor functions. A more recent model was characterized by Prediger and colleagues who administered MPTP to rodents via an intranasal infusion (Prediger et al. 2011, 2010, 2006). Intranasal MPTP delivery resulted in olfactory, cognitive, emotional and motor defects, loss of TH-ir cells in the olfactory bulb and SNpc, decreased fiber density in the striatum as well as dopamine depletion in the olfactory bulb, striatum and prefrontal cortex. These features appear analogous to many of those observed during the prodromal and early stages of PD making the model a valuable tool for testing novel therapeutic strategies to restore sensory and cognitive deficits of early PD. The MPTP monkey model, however, remains the gold standard model in the preclinical assessment of novel treatment strategies as a final step before proceeding to clinical trials (Burns et al. 1983; Emborg 2007; Porras et al. 2012).

Rotenone is a natural cytotoxic compound used as an insect and fish poison. Systemically administered rotenone reproduces many features of PD but can cause high mortality in experimental animals (Heikkila et al. 1985b; Betarbet et al. 2000; Gubellini and Kachidian 2015). The dopaminergic damage caused by rotenone can be variable or difficult to reproduce. Paraquat (N,N'-dimethyl-4-4'-bipiridinium), a structural analog of MPP+, is a potent and widely used herbicide that produces progressive, but poorly reproducible, dopaminergic neurodegeneration and accumulation of α-syn immunoreactive inclusions after systemic administration (Cochemé

and Murphy 2008; Manning-Bog et al. 2002; McCormack et al. 2002; Ossowska et al. 2005; Rappold et al. 2011; Richardson et al. 2005; Shimizu et al. 2001).

Lactacystin is a selective proteasome inhibitor. When stereotaxically injected of into the nigrostriatal pathway, it disturbs cellular protein homeostasis, causes dose-dependent formation of cytoplasmic, α-syn and ubiquitin containing protein inclusions and triggers dopaminergic neurodegeneration (McNaught et al. 2002b; Bentea et al. 2017). Other proteasome inhibitors, for example systemic administration of PSI (*N*-carbobenzoxy-L-isoleucyl-L-γ-*t*-butyl-L-gultamyl-L-alanyl-L-leucinal), have also been tested to model PD in rodents and NHPs with equivocal results (Bentea et al. 2017).

2.4.3 Genetic models

A number of the genetic causes of familial PD have been modeled in mice and rats (Dawson et al. 2010). The most common genetic models are derived from transgenic overexpression of autosomal dominant genes (*SNCA* and *LRRK2*) or knockout of autosomal recessive genes (*PRKN*, *PINK1* and *DJ-1*). Even though these models represent direct etiological causes of PD, and thus should replicated relevant pathogenic mechanisms, they fail to produce robust nigrostriatal degeneration, striatal dopamine depletion or motor impairments. Nevertheless, they provide valuable tools to dissect the pathophysiological roles of these proteins.

Duplication or triplication of *SNCA* gene leads to a rare form of familial early onset PD with a clear gene dosage effect on the severity of the disease. This suggests that the level of α-syn dictates its toxicity and has spurred the creation of several animal models based on the overexpression of human or murine wild-type α-syn (Dawson et al. 2010; Visanji et al. 2016). Other α-syn transgene models overexpress PD-related missense mutations of *SNCA* (e.g. A30P or A53T). Viral vectors, such as adeno-associated virus (AAV) or lentivirus, offer another approach to deliver α-syn transgene into the brain of wild-type animals which has been shown to recapitulate the essential neuropathological features of PD (Kirik et al. 2003, 2002; Lo Bianco et al. 2002). It is worthwhile to note, however, that transgenic α-syn models rely on the expression of supraphysiological levels of α-syn which might reduce their validity in mimicking sporadic PD pathology.

Knockout of *PRKN*, *PINK1* or *DJ-1* genes seems to be insufficient to perturb the survival or function of nigrostriatal dopamine neurons or to induce formation of α-syn positive protein inclusions (Dawson et al. 2010; Blesa and Przedborski 2014). Due to the absence of consistent parkinsonian phenotype in single knockout mice, a triple knockout model with parkin, DJ-1 and PINK1 deletion was created (Kitada et al. 2009). However, even the inactivation of all three recessive PD genes was insufficient to induce loss of nigral dopamine neurons within the mouse lifespan. Only bacterial artificial chromosome (BAC) -mediated overexpression of truncated human *PRKN* (Q311X) in dopamine neurons has been shown to produce age-dependent degeneration of nigrostriatal dopamine system, accumulation of α-syn positive protein inclusions and late-onset motor deficits in mice (Lu et al. 2009).

MitoPark is an example of a genetic PD model that is not derived from any of the known PD-related genetic risk factors. In MitoPark mice, *TFAM* gene encoding mitochondrial transcription factor A (TFAM) is conditionally deleted from dopaminergic neurons (Ekstrand et al. 2007; Galter et al. 2010; Good et al. 2011). TFAM is required for the transcription of mitochondrial DNA coding for essential subunits of the respiratory chain. MitoPark mice show apparent PD-like phenotype both on pathological and behavioral level.

2.4.4 Alpha-synuclein pathology spreading model

Recombinant α-syn preformed fibrils (PFFs) can be inoculated into various brain regions to induce propagating Lewy body-like pathology both in mice with different genetic backgrounds, rats and NHPs (Luk et al. 2012; Luk et al. 2012b; Rey et al. 2016; Abdelmotilib et al. 2017; Karampetsou et al. 2017; Thakur et al. 2017; Shimozawa et al. 2017; Chu et al. 2019; Henderson et al. 2019). PFF-induced α-syn pathology triggers reactive microgliosis prior to dopaminergic degeneration in the SNpc suggesting that neuroinflammation contributes the vulnerability of dopamine neurons (Duffy et al. 2018). One advantage of this model is that the PD-like phenotype develops slower than in neurotoxin or AAV-α-syn overexpression models offering a therapeutic window for experimental interventions (Okuzumi et al. 2018).

2.4.5 Behavioral and histological assessment in animal models

As described in chapter 2.2, the nigrostriatal dopamine system plays a key role in regulating motor behavior. Thus, the degree of nigrostriatal degeneration in animal models of PD can be followed longitudinally by measuring motor deficits with specific behavioral tests (Asakawa et al. 2016; Dunnett and Lelos 2010). The behavioral tests that are commonly used to measure motor impairment in rodents include, but are not restricted to, drug-induced rotational behavior (rotametry), limb-use asymmetry (cylinder), open field, rotarod, stepping, skilled paw-reaching (staircase) and adhesive removal tests. The non-motor symptoms of PD can also be measured with various behavioral tests which are not, however, specifically designed for rodent models of PD. The brains of NHPs are anatomically and physiologically closest to the human brains making NHPs invaluable subjects in PD research (Emborg 2007; Porras et al. 2012). NHPs replicate almost all of the human parkinsonian motor symptoms after neurotoxin administration enabling much more versatile behavioral assessments as compared to other species, for example the use of various rating scales that imitate the clinically used Unified Parkinson's disease Rating Scale (UPDRS).

When studying disease-modifying therapies in animal models of PD, the decisive neuropathological outcomes (e.g. neuroprotective or restorative effects) can be obtained only with cellular and molecular level investigation after tissue collection. Histologically, the integrity of the nigrostriatal pathway can be measured by immunolabelling dopaminergic markers such as TH, DAT or VMAT2 and quantifying the number of immunoreactive cell bodies in the SNpc and the density of immunoreactive fibers in the dorsal striatum. Lewy body-like pathology can be visualized for example by immunostaining phosphorylated α-syn. In histological assessments, it is important to note that the expression levels of individual molecular markers are under divergent

regulation. Thus, a combination of markers should be favored in order to draw reliable conclusions.

2.5 Preclinical studies of neurotrophic factors for Parkinson's disease

Due to the dopaminotrophic properties of GDNF, NRTN, CDNF and MANF, their potential as a disease-modifying therapy for PD has been examined extensively both *in vitro* and *in vivo*. Here, an overview of the key preclinical studies, with the main focus on *in vivo* experiments in normal and lesioned nigrostriatal dopamine system, will be provided.

2.5.1 Effects of neurotrophic factors on dopaminergic function of intact nigrostriatal system

NTF delivery into the brain of a PD patient would be likely to affect both degenerating and intact neurons. Therefore, knowledge on the effects of exogenous recombinant NTFs not only on the pathological processes but also on the physiological functions of the brain is pivotal. Characterization of the effects of NTFs on normal, non-lesioned basal ganglia circuitry allows for improving their therapeutic efficacy and safety profile.

2.5.1.1 Neurochemical and functional effects of exogenous GDNF

The exogenous administration of GDNF elicits long-lasting neurochemical and functional changes in the intact nigrostriatal system. GDNF augments dopamine, DOPAC and HVA levels in tissue samples and induces spontaneous motor activity in young and aged animals (Hudson et al. 1995; Hebert et al. 1996; Martin et al. 1996; Hebert and Gerhardt 1997; Kobayashi et al. 1998; Grondin et al. 2003; Georgievska et al. 2004; Hadaczek et al. 2010). Dopamine turnover, measured as HVA/dopamine ratio, reflects the strength of dopaminergic transmission and is enhanced in GDNF-treated animals. The abovementioned effects, however, are not specific to the dopaminergic system, since nigrostriatal serotonin, 5-hydroxyindoleacetic acid (5-HIAA) and noradrenaline levels are also altered. Intracranial GDNF injections are also associated with reduced food intake and body weight in experimental animals. Noteworthily, unilateral striatal delivery of GDNF has been shown to produce bilateral effects on dopamine neurochemical markers, neuronal activity and motor functions in normal animals as well as in PD patients (Grondin et al. 2003; Salvatore et al. 2009; Slevin et al. 2005; Stanford et al. 2007).

GDNF has been shown to modulate the activity of dopaminergic nerve terminals. GDNF application increases stimulus-evoked dopamine release in cultured midbrain dopamine neurons as well as in striatal synaptosomes and slices (Feng et al. 1999; Gomes et al. 2006). Amperometric recordings showed that in primary cultures GDNF exposure increased the quantal size of dopamine-containing synaptic vesicles (Pothos et al. 1998). With patch clamp technique, GDNF was demonstrated to acutely potentiate the excitability and synaptic transmission of dopamine neurons in midbrain cultures and slices by inhibiting A-type K^+ channels and potentiating high voltage-gated Ca^{2+} channel currents (Bourque and Trudeau 2000; Yang et al. 2001; Wang et al.

2003). Similar effects have also been observed *in vivo* in brain microdialysis measurements. Intracranial administration of GDNF augmented stimulus-evoked overflow of striatal dopamine in non-lesioned rats (Hebert et al. 1996; Hebert and Gerhardt 1997; Cass et al. 1999; Salvatore et al. 2004; Cass and Peters 2010) and NHPs (Gash et al. 1995; Grondin et al. 2003). These microdialysis studies, however, were conducted under general anesthesia which is known to have profound effects on neuronal activity, neurotransmitter synthesis, release, reuptake and metabolism (Marinelli and McCutcheon 2014; Müller et al. 2011). In a microdialysis study with freely-moving rats, no significant differences in potassium-evoked dopamine output was detected between GDNF- and vehicle-injected animals (Xu and Dluzen 2000).

GDNF-induced enhancements in dopaminergic function are associated with a sustained increase in TH phosphorylation and activity. Exogenous GDNF has been shown to downregulate the expression of TH but increase its phosphorylation and activity (Beck et al. 1996; Rosenblad et al. 2003; Georgievska et al. 2004; Kobori et al. 2004; Salvatore et al. 2004, 2009). The downregulation of TH may be a compensatory response to its enhanced activity. In line with this, *in vivo* TH activity is also elevated in MEN2B (multiple endocrine neoplasia type 2B) mice with constitutively active RET (Mijatovic et al. 2008).

2.5.1.2 Neurochemical and functional effects of other NTFs

Data on the molecular and functional effects of other exogenously administered GFLs, CDNF or MANF on the intact nigrostriatal system are scarce. Similarly to GDNF, striatal administration of NRTN has been shown to provoke some behavioral and neurochemical changes associated with functional upregulation of dopamine neurons. In non-lesioned rats, NRTN delivery augmented amphetamine-induced locomotor activity and caused a persistent increase in dopamine turnover (Horger et al. 1998; Hadaczek et al. 2010). A single nigral injection of NRTN increased stimulus-evoked dopamine release in the striatum and tissue levels of dopamine in the SN of normal rats (Cass and Peters 2010). In naïve monkeys, striatal delivery of an AAV2 vector encoding human NRTN (CERE-120, see Table 2.3) increased the number of TH-ir cells in the SNpc and fibers in the striatum (Herzog et al. 2008, 2007). PET imaging demonstrated that ^{18}F-dopa uptake was increased in the NTRN-transduced striatum as compared to the control side.

2.5.1.3 Diffusion properties of GDNF, NRTN, CDNF and MANF in the intact brain

The volume of distribution of exogenous GDNF and NRTN in the brain parenchyma is limited due to their high-affinity binding to extracellular matrix-associated heparan sulfates (Hamilton et al. 2001; Piltonen et al. 2009; Runeberg-Roos et al. 2016). Poor diffusion of GDNF has also been demonstrated in the normal rhesus monkey brain (Lapchak et al. 1998; Salvatore et al. 2006). Intrastriatally administered MANF, instead, diffuses significantly better than GDNF in the intact rat brain distributing throughout the striatum and frontal cortex (Voutilainen et al. 2011, 2009). CDNF also diffuses rapidly in the intact rat brain resulting in widespread distribution that extends almost over the entire hemisphere at the level of the striatal infusion site (Mätlik et al. 2017).

2.5.2 Effects of neurotrophic factors in animal models of Parkinson's disease

According to the initial findings, GDNF selectively promotes the survival and morphological differentiation of dopamine neurons in rat embryonic midbrain cultures and enhances their high-affinity dopamine uptake (Lin et al. 1993). GDNF is also able to support the viability of nigral dopamine neurons in postnatally derived primary cultures by inhibiting natural cell death (Burke et al. 1998). In *in vitro* neurotoxin models, GDNF prevents the loss of cultured midbrain dopamine neurons in response to MPP+ or 6-OHDA (Eggert et al. 1999) and promotes the recovery and regrowth of damaged dopamine neurons after the removal of the toxin challenge (Hou et al. 1996; Kramer et al. 1999). Likewise, NRTN supports the survival of dopamine neurons in embryonic midbrain cultures with similar potency and efficacy to that observed for GDNF (Cacalano et al. 1998; Horger et al. 1998; Runeberg-Roos et al. 2016). MANF was shown to selectively increase the survival and sprouting of dopamine neurons in embryonic midbrain cultures (Petrova et al. 2003). CDNF protected primary neurons in embryonic midbrain cultures against cytotoxicity induced by 6-OHDA and α-syn oligomers (Latge et al. 2015). The promising outcomes in *in vitro* studies have encouraged testing the neuroprotective and regenerative effects of GDNF, NRTN, CDNF and MANF in various animal models of PD. An overview of these *in vivo* studies is provided in Table 2.3.

Notably, according to toxin-induced neurorestoration models, GDNF delivery into the striatum seems to provide better protection on TH-ir fibers in the striatum and result in more robust functional recovery as compared to nigral delivery. GDNF delivery to its physiological site of expression (i.e. the striatum) may be an underlying cause for this discrepancy (Kirik et al. 2004; Ibáñez and Andressoo 2017).

A combined lentiviral vector-mediated transduction of CDNF and MANF into the SNpc led to synergistic neuroprotective effects in 6-OHDA-lesioned rats with reduced amphetamine-induced turning behavior and robust preservation of dopaminergic fibers in the striatum and cell bodies in the SNpc (Cordero-Llana et al. 2015c). Also, when CDNF protein was injected into the striatum of 6-OHDA-lesioned rats together with GDNF, the co-administration resulted in stronger neurorestorative effects than either of the NTFs could produce alone (Voutilainen et al. 2017). The additive effects of the NTFs may stem from different mechanisms of action: both proteins activated the survival promoting PI3K/Akt signaling pathway and CDNF reduced the expression of ER stress-related markers GRP78, ATF6 and phosphorylated eIF2α.

Continuous NTF infusion may cause ligand-dependent receptor desensitization decreasing the responsiveness of the target tissue to the treatment (Lohse 1993). To circumvent this risk, intermittent convection-enhanced delivery (CED), which enables target engagement in a pulsatile fashion, has been developed and tested in preclinical studies (Bobo et al. 1994; Gash et al. 2005). CED significantly enhances tissue distribution of macromolecules by delivering brief bolus infusions of a treatment solution in addition to low-rate basal infusion.

Table 2.3. Disease-modifying effects of neurotrophic factors (NTFs) in animal models of Parkinson's disease (PD).

NTF	Species; PD model	Para-digm[a]	NTF delivery	NTF dose	Neuropathological and neurochemical outcomes	Functional outcomes	Reference
GDNF	Rats; Unilateral 6-OHDA-lesion in MFB	Neuro-restoration	Single ipsilateral injection into SN	100 μg	Preserved nigral DA and metabolite levels; increased survival of TH+ neurons and neurites in SN	Reduced APO-induced rotations	(Hoffer et al. 1994); (Bowenkamp et al. 1995)
	Mice; Repeated MPTP s.c. injections	Neuro-protection	Single unilateral injection into SN or STR	10 μg	Preserved nigrostriatal DA and metabolite levels, increased survival of TH+ neurons in SN and TH+ fibers in STR	Increased locomotor activity	(Tomac et al. 1995)
		Neuro-restoration			Partially preserved nigrostriatal DA and metabolite levels, increased survival TH+ fibers in STR		
	Rats; Unilateral 6-OHDA-lesion in STR	Neuro-protection	Single ipsilateral injection into SN, STR or LV	25 μg, 25 μg or 50 μg	Increased survival of TH+ neurons in SN (STR and SN delivery), increased survival TH+ fibers in STR (STR delivery)	Normalized contralateral forelimb akinesia in stepping test, reduced APO- and AMPH-induced rotations (STR delivery)	(Kirik et al. 2000)
	Rats; Unilateral 6-OHDA-lesion in STR	Neuro-protection	AAV vector-mediated ipsilateral transduction into SN, STR or SN+STR	$4x10^9$ vg (SN), $9x10^9$ vg (STR)	Increased survival of TH+ neurons in SN (SN, STR and SN+STR delivery), increased survival TH+ fibers in STR (STR delivery)	Reduced AMPH-induced rotations, improved spontaneous contralateral paw use in cylinder and staircase test (STR delivery)	(Kirik et al. 2000b)
	Rats; Unilateral 6-OHDA-lesion in SN	Neuro-restoration	Lentiviral vector-mediated ipsilateral transduction into STR	$2.6x10^5$ TU	Increased survival TH+ fibers in STR	Improved spontaneous contralateral paw use in staircase test	(Brizard et al. 2006)
	Rats; Unilateral 6-OHDA-lesion in STR	Neuro-protection	Single ipsilateral injection into STR	10 μg	Increased survival of TH+ neurons in SN and TH+ fibers in STR	Reduced AMPH-induced rotations	(Yue et al. 2014)
		Neuro-restoration			Increased survival of TH+ neurons in SN		
	Rats; Unilateral lentiviral vector-transduction of A30P hα-syn to SN	Neuro-protection	Lentiviral vector-mediated ipsilateral transduction into SN	?	Lack of protective effect on TH+ neurons in SN and TH+ fibers in STR	N.D.	(Lo Bianco et al. 2004a)
	Rats; Unilateral AAV vector-transduction of wild-type hα-syn to SN	Neuro-protection	Lentiviral or AAV vector-mediated ipsilateral transduction into SN, STR or SN+STR	$3x10^4$ TU (SN), $4.5x10^4$ TU (STR)	Lack of protective effect on TH+ neurons in SN and TH+ and VMAT2+ fibers in STR, no effect on the number of α-syn+ aggregates	Failed to reduce AMPH-induced rotations	(Decressac et al. 2011)
	Monkeys; Unilateral 6-OHDA-lesion in MFB	Neuro-protection	AAV vector-mediated ipsilateral transduction into SN and STR	$2.4x10^9$ vg (SN), $6x10^9$ vg (STR)	Increased survival of TH+ and VMAT2+ neurons in SN, increased size of TH+ neurons in SN	Improved rating scale scores, reduced AMPH- and APO-induced rotations	(Eslamboli et al. 2003)
	Monkeys; Unilateral MPTP infusion via car. art.	Neuro-restoration	Ipsilateral injection into SN, STR or LV	1x150 μg, 1x450 μg or 3x100/450 μg	Increased cell size and neurite density of TH+ neurons in SN (SN and STR delivery), preserved DA levels in ventral midbrain (ICV delivery)	Improved rating scale scores in bradykinesia, rigidity, posture and balance	(Gash et al. 1996)
	Monkeys; Unilateral MPTP infusion via car. art.	Neuro-restoration	Single ipsilateral injection into LV	300 μg	Preserved nigrostriatal DA and metabolite levels	Improved rating scale scores	(Gerhardt et al. 1999)
	Monkeys; Unilateral MPTP infusion via car. art.	Neuro-restoration	Lentiviral vector-mediated ipsilateral transduction into SN+STR	?	Increased survival of TH+ neurons in SN and TH+ fibers in STR	Increased uptake in ^{18}F-DOPA-PET, improved rating scale scores, reversed motor deficits in a hand-reach task	(Kordower et al. 2000); (Emborg et al. 2009)
	Monkeys; Unilateral MPTP infusion via car. art.	Neuro-restoration	Chronic infusion to ipsilateral LV or bilaterally into STR	5 or 15 μg/24h	Increased cell size and survival of TH+ neurons in SN, increased survival TH+ fibers in STR, preserved DA and metabolite levels in STR and GP	Improved rating scale scores in bradykinesia, rigidity, posture and balance	(Grondin et al. 2002)
	Monkeys; Unilateral MPTP infusion via car. art.	Neuro-restoration	Pulsed, CED infusion into ipsilateral SN	7.5 or 22.5 μg/24h	Increased cell size and survival of TH+ neurons in SN	Improved rating scale scores, increased movement speed	(Gash et al. 2005)
NRTN	Rats; Unilateral 6-OHDA-lesion in STR	Neuro-restoration	Single or repeated ipsilateral injection into SN	1x1 or 1x10 μg or 7x5 μg (every 3rd day)	Increased survival of TH+ (single injection) and FG+ neurons in SN (single and repeated injections)	N.D.	(Horger et al. 1998)
	Rats; Unilateral 6-OHDA-lesion in STR	Neuro-restoration	Multiple ipsilateral injections into STR or LV	7x5 or 7x10 μg (every 3rd day)	Increased survival of TH+ and FG+ neurons in SN (STR delivery)	No effect on AMPH-induced rotations	(Rosenblad et al. 1999)

	Rats; Unilateral 6-OHDA-lesion in STR	Neuro-protection Neuro-restoration	Single ipsilateral injection into STR	5 μg	Increased survival of TH+ neurons in SN, preserved striatal DA and metabolite levels Increased survival TH+ fibers in STR, preserved striatal DA and metabolite levels	Reduced MetAMPH-induced rotations, increased DA turnover (restoration study)	(Oiwa et al. 2002)
	Rats; Unilateral 6-OHDA-lesion in STR	Neuro-protection	AAV2 vector (CERE-120[b]) -mediated ipsilateral transduction into STR	1.6x10^8, 8x10^8 or 4x10^9 vg	Increased survival of TH+ neurons in SN, increased survival of VMAT2+ fibers in STR	Reduced AMPH-induced rotations, no effect on spontaneous forepaw use in the cylinder test	(Gasmi et al. 2007) (Gasmi et al. 2007b)
	Monkeys; Unilateral MPTP infusion via car. art.	Neuro-restoration	AAV2 vector (CERE-120[b]) -mediated ipsilateral transduction into SN+STR	Total dose 1.7x10^{11} TU	Increased survival of TH+ neurons in SN and TH+ fibers in STR	Improved rating scale scores	(Kordower et al. 2006)
	Monkeys; Unilateral MPTP infusion via car. art.	Neuro-restoration	Chronic infusion into ipsilateral STR	30 μg/24h	Preserved DA metabolite levels in GP	Improved rating scale scores, increased locomotor activity	(Grondin et al. 2008)
CDNF	Rats; Unilateral 6-OHDA-lesion in STR	Neuro-protection Neuro-restoration	Single ipsilateral injection into STR	1-10 μg 10 μg	Increased survival of TH+ neurons in SN and TH+ fibers in STR (3-10 μg) Partially (N.S.) increased survival TH+ neurons in SN	Reduced AMPH-induced rotations (3-10 μg)	(Lindholm et al. 2007)
	Rats; Unilateral 6-OHDA-lesion in STR	Neuro-restoration	Chronic infusion into ipsilateral STR	1.5-4.5 μg/24h	Increased survival of TH+ neurons in SN (3-4.5 μg/24h) and TH+ fibers in STR (1.5-4.5 μg/24h)	Reduced AMPH-induced rotations (3 μg/24h)	(Voutilainen et al. 2011)
	Mice; Repeated MPTP i.p. injections	Neuro-protection Neuro-restoration	Single bilateral injections into STR	10 μg/side	Increased survival of TH+ neurons in SN and TH+ fibers in STR	Increased locomotor activity	(Airavaara et al. 2012)
	Rats; Unilateral 6-OHDA-lesion in STR	Neuro-protection	AAV vector-mediated ipsilateral transduction into STR	4x10^7, 2x10^8 or 1x10^9 vg	Partially (N.S.) increased survival TH+ neurons in SN (2x10^8 and 1x10^9 vg), partially (N.S.) increased survival TH+ fibers in STR (1x10^9 vg)	Reduced AMPH-induced rotations (2x10^8 and 1x10^9 vg)	(Back et al. 2013)
	Rats; Unilateral 6-OHDA-lesion in STR	Neuro-restoration	AAV vector-mediated ipsilateral transduction into STR	?	Increased survival of TH+ neurons in SN and TH+ fibers in STR, preserved TH expression in SN, preserved TH and DAT expression in STR	Reduced AMPH-induced rotations, increased locomotor activity, increased DAT-PET uptake in STR	(Ren et al. 2013)
	Rats; Unilateral 6-OHDA-lesion in STR	Neuro-protection	Lentiviral vector-mediated ipsilateral transduction into SN or STR	6x10^5 TU	Increased survival of TH+ fibers in STR (SN delivery), lack of protective effect on TH+ neurons in SN (SN and STR delivery) and TH+ fibers in STR (STR delivery)	Reduced AMPH-induced rotations (SN delivery), no effect on AMPH- or APO induced rotations (STR delivery)	(Cordero-Llana et al. 2015c)
	Rats; Unilateral 6-OHDA-lesion in MFB	Neuro-restoration	Single ipsilateral injection into SN	1-100 μg	No effect on TH+ neurons in SN and TH+ fibers in STR	Modestly reduced APO- induced rotations (10 μg)	(Huotarinen et al. 2018)
	Monkeys; Mild unilateral 6-OHDA-lesion in STR	Neuro-restoration	Ipsilateral injection into STR (two injection sites)	2x10 μg	No effects on TH+ fibers or neurons	Increased DAT-SPECT uptake in STR (proper negative control group and between group comparisons are lacking)	(Garea-Rodríguez et al. 2016)
MANF	Rats; Unilateral 6-OHDA-lesion in STR	Neuro-protection Neuro-restoration	Single ipsilateral injection into STR	3-30 μg 10 μg	Increased survival of TH+ neurons in SN (10 μg) Partially (N.S.) increased survival TH+ neurons in SN	Reduced AMPH-induced rotations (10 μg)	(Voutilainen et al. 2009)
	Rats; Unilateral 6-OHDA-lesion in STR	Neuro-restoration	Chronic infusion into ipsilateral STR	1.5-4.5 μg/24h	Lack of protective effect on TH+ neurons in SN and TH+ fibers in STR	No effect on AMPH-induced rotations	(Voutilainen et al. 2011)
	Rats; Unilateral 6-OHDA-lesion in STR	Neuro-protection	Lentiviral vector-mediated ipsilateral transduction into SN or STR	1.6x10^6 TU	Increased survival of TH+ neurons in SN (SN delivery), lack of protective effect on TH+ neurons in SN (STR delivery) and TH+ fibers in STR (SN and STR delivery)	No effect on AMPH- or APO induced rotations	(Cordero-Llana et al. 2015c)
	Rats; Unilateral 6-OHDA-lesion in STR	Neuro-restoration	AAV vector-mediated ipsilateral transduction into STR	2x10^{10} vg	Increased survival of TH+ neurons in SN and TH+ fibers in STR, increased striatal DA and metabolite levels	Reduced AMPH-induced rotations	(Hao et al. 2017)

AAV, adeno-associated virus; AMPH, amphetamine; APO, apomorphine; car. art., carotid artery; CED, convection-enhanced delivery; DA, dopamine; DAT, dopamine transporter; FG, fluorogold; GP, glopus pallidus; hα-syn, human α-synuclein; i.p., intraperitoneal; LV, lateral ventricle; MFB, medial forebrain bundle; N.D., not determined; N.S., not significant; s.c., subcutaneous; SN, substantia nigra; STR, striatum; TH, tyrosine hydroxylase; TU, transducing units (lentiviral titer); vg, viral genomes (AAV titer); VMAT2, vesicular monoamine transporter 2

[a] In neuroprotection paradigm, NTF is administered prior to, or at the same time as the lesion. A neuroprotective therapy slows down or prevents neurodegeneration. In neurorestoration paradigm, NTF is applied after the lesion when neuronal damage has occurred. Neurorestoration refers to recovery of damaged or impaired neuronal connections, somas, functionality or phenotype.

[b] CERE-120 is an AAV2-derived vector encoding a hybrid form of human NRTN cDNA where the NRTN pre-pro domain is replaced with that of human NGF to ensure enhanced secretion (Ramirez et al. 2004; Gasmi et al. 2007)

2.6 Neurotrophic factors in clinical trials for Parkinson's disease

The encouraging outcomes in preclinical studies spurred the assessment of intracerebrally delivered recombinant NTFs in PD patients. To date, four different trophic factors have entered clinical trials in PD: GDNF, NRTN, platelet-derived growth factor (PDGF) and most recently CDNF. A summary of these trials is provided in Table 2.4., excluding the placebo-controlled phase I/II study by Paul et al. (2015) assessing the safety and tolerability of intracerebroventricularly (ICV) infused PDGF-BB, which lies beyond the scope of this thesis.

2.6.1 Clinical studies with GDNF

The safety and efficacy of GDNF protein therapy as well as AAV2 vector -mediated gene therapy have been evaluated in several clinical trials in patients with advanced PD (Table 2.4.). In two separate open-label phase I trials, intraputamenal infusion of GDNF led to significantly improved motor function associated with increased ^{18}F-DOPA uptake in the posterior putamen after one year of treatment and caused no serious adverse effects (Gill et al. 2003; Patel et al. 2005; Slevin et al. 2007, 2005). A case report described that these functional improvements sustained up to three years after the cessation of the protein infusion (Patel et al. 2013). Nevertheless, the primary endpoints in two double-blind, placebo-controlled phase I/II trials were not met (Nutt et al. 2003; Lang et al. 2006). ICV injections of GDNF failed to improve patients' UPDRS scores and caused severe side effects like nausea, vomiting, weight loss, anorexia and paresthesias (Nutt et al. 2003). The lack of efficacy was possibly due to a suboptimal delivery method; a *postmortem* histological analysis of one GDNF-treated subject from the study suggested the lack of target engagement by showing little penetration of GDNF into the brain parenchyma from the lateral ventricle (Kordower et al. 1999). Likewise, intraputamenal infusion of GDNF was unable to improve the off-medication UPDRS motor score although ^{18}F-DOPA uptake was increased in GDNF-infused patients after six months of treatment (Lang et al. 2006). Intraputamenal infusion of GDNF, however, seemed to be better tolerated as compared to ICV injections: treatment-related adverse events included mainly paraesthesias, headache and upper respiratory tract infections in some subjects. In a few patients, neutralizing antibodies against the recombinant GDNF were detected.

The discrepancies in the outcomes of these first trials could be attributed to insufficient bioavailability of GDNF in the target area (Sherer et al. 2006). The continuous low-rate intraputamenal infusion protocol utilized in Lang et al. (2006) study resulted in spatially limited and heterogenous distribution in the putamen (Salvatore et al. 2006). Intermittent intraputamenal CED delivery, instead, allows much wider and homogeneous exposure across the putamen and avoids receptor desensitization (Bobo et al. 1994; Lohse 1993). Thus, Whone and colleagues set out to evaluate the efficacy and safety of recombinant GDNF administered via intermittent intraputamenal CED infusion in a randomized, placebo-controlled phase II study (Whone et al. 2019; Whone et al. 2019b). Although there was a clinically significant motor improvement in 43% of the GDNF-treated patients and overall increase in putamenal ^{18}F-DOPA uptake in the GDNF

group, the trial failed to meet its primary efficacy endpoint. The treatment administration setup used in this trial is illustrated in Figure 2.12.

Table 2.4. Neurotrophic factors (NTFs) in clinical trials for Parkinson's disease (PD).

NTF	Delivery	Dosing	Phase / design	Patients	Key outcomes	References
GDNF	rhGDNF; lateral ventricle; unilateral bolus injections 1/month	Dose-escalation: 25, 75, 150, 300 or 4000 µg for 6-8 months + 20 months open-label extension	I/II, randomized, double-blind, placebo-controlled	50; moderate to severe PD H&Y 3-5 (off)	No improvement in UPDRS motor scores, various SAEs e.g. paresthesias, nausea, anorexia, weight loss and vomiting	(Nutt et al. 2003)
	rhGDNF; putamen; uni- or bilateral continuous infusion	11–43 µg/putamen/24h for 12 months + 12 months extension at 14-29 µg/putamen/24h	I, open-label	5; advanced PD (>6 years from diagnosis)	57% improvement in UPDRS motor score (off) at 24 months, increased ^{18}F-DOPA-PET uptake in putamen, sprouting of dopaminergic fibers, no therapy-related SAEs	(Gill et al. 2003) (Patel et al. 2005) (Love et al. 2005)
	rhGDNF; putamen; unilateral CED infusion	Dose-escalation: 3, 10 and 30 µg/day for 6 months + 12 months extension at 30 µg/24h	I, open-label	10; moderate to severe PD H&Y 3-4 (off)	45% bilateral improvement in UPDRS motor score (off) at 12 months, no therapy-related SAEs, anti-GDNF antibodies in some patients	(Slevin et al. 2005) (Slevin et al. 2007)
	rhGDNF; putamen; bilateral continuous infusion	15 µg/putamen/24h for 6 months + open-label extension	II, randomized, double-blind, placebo-controlled	34; advanced PD (>5 years from diagnosis)	No improvement in UPDRS motor score (off), increased ^{18}F-DOPA-PET uptake in putamen, well tolerated, anti-GDNF antibodies in some patients	(Lang et al. 2006)
	rhGDNF; putamen; bilateral intermittent CED infusions 1/month	120 µg/putamen for 9 months + 9 months open-label extension	II, randomized, double-blind, placebo-controlled	41; moderate PD H&Y 2-3 (off)	No improvement in UPDRS motor score (off), increased ^{18}F-DOPA-PET uptake in putamen, no therapy-related SAEs	(Whone et al. 2019) (Whone et al. 2019b) NCT03652363[a]
	AAV2-GDNF; putamen; bilateral CED infusion	Dose-escalation: $9x10^{10}$, $3x10^{11}$ or $9x10^{11}$ vg	I, open-label	13; moderate to severe PD H&Y 3-4 (off)	No improvement in UPDRS motor scores, increased ^{18}F-DOPA-PET uptake in putamen, no therapy-related SAEs, anti-GDNF and anti-AAV2 antibodies in some patients	(Heiss et al. 2019) NCT01621581[a]
NRTN	AAV2-NRTN[b]; putamen; bilateral multi-site injections	Dose-escalation: $1.3x10^{11}$ or $5.4x10^{11}$ vg	I, open-label	12; moderate to severe PD H&Y 3-4 (off)	36% improvement in UPDRS motor score (off) at 12 months, no increase in ^{18}F-DOPA-PET uptake, no therapy-related SAEs	(Marks et al. 2008) NCT00252850[a]
	AAV2-NRTN[b]; putamen; bilateral multi-site injections	$5.4x10^{11}$ vg + double-blind extension up to 18 months (n=30)	II, randomized, double-blind, sham-surgery controlled	58; advanced PD (>5 years from diagnosis)	No improvement in UPDRS motor score (off) at 12 months, no increase in ^{18}F-DOPA-PET uptake, improved PDQ-39 score at 12 months, improved UPDRS motor score (off) at 18 months (n=30), no therapy-related SAEs	(Marks et al. 2010) NCT00400634[a]
	AAV2-NRTN[b]; putamen+SN; bilateral multi-site injections	Dose-escalation: $2x10^{11}$ vg/SN + $2.7x10^{11}$ vg/putamen or $2x10^{11}$ vg/SN + $1.2x10^{12}$ vg/putamen	I, open-label	6; moderate PD H&Y 2-3 (off)	Well-tolerated, no therapy-related SAEs	(Bartus et al. 2013) NCT00985517[a]
	AAV2-NRTN[b]; putamen+SN; bilateral multi-site injections	$2x10^{11}$ vg/SN + $1x10^{12}$ vg/putamen	II, randomized, double-blind, sham-surgery controlled	51; moderate PD H&Y 2-3 (off)	No improvement in UPDRS motor score (off), no therapy-related SAEs, anti-AAV2 antibodies in some patients	(Olanow et al. 2015) NCT00985517[a]
CDNF	rhCDNF; putamen; intermittent CED infusions 1/month	Low-dose or high-dose for 6 months + 6 months open-label extension	I/II, randomized, double-blind, placebo-controlled	17; moderate PD H&Y 2.5-3 (off)	Well-tolerated, no treatment-related SAEs, potentially improved UPDRS motor score in some patients at 12 months, increased DAT-PET uptake in putamen of some patients, ongoing analyses of exploratory endpoints	(Huttunen and Saarma 2019) NCT03295786[a]

AAV2, adeno-associated virus serotype 2; CED, convection-enhanced delivery; H&Y, Hoehn and Yahr scale of Parkinson's disease symptoms; off, off medication; PDQ-39, a 39-item Parkinson's disease Questionnaire to assess health and quality of life; rh, recombinant human; SAE, serious adverse event; SN, substantia nigra; UPDRS, Unified Parkinson's Disease Rating Scale; vg, viral genomes
[a] ClinicalTrials.gov identifier; [b] CERE-120

Viral vector -mediated gene transfer provides another opportunity for targeted delivery of therapeutic proteins into restricted brain regions in a safe and sustained manner. Gene therapy

may also enhance the tissue distribution of the expressed protein. An open-label phase I trial sought to assess the safety and effectiveness of bilateral intraputamenal AAV2-GDNF gene therapy in advanced PD (Heiss et al. 2019). Delivery of AAV2-GDNF using intraoperative MRI-guided CED infusion was safe and well tolerated. It also increased ^{18}F-DOPA uptake in the putamen suggesting a neurotrophic effect on dopaminergic neurons but failed provide any clinically significant improvements in the UPDRS scores.

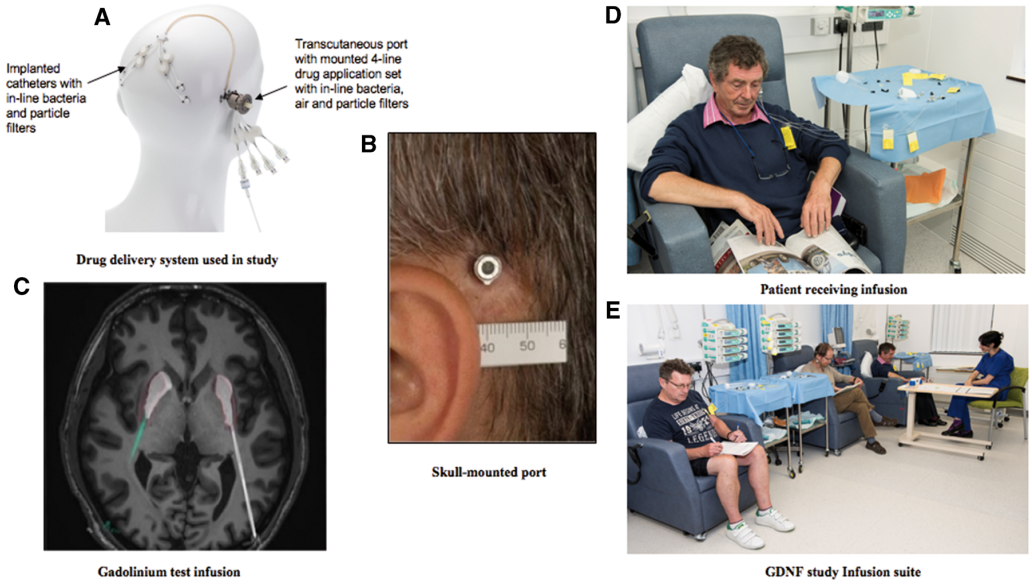

Figure 2.12. Intracranial administration of GDNF to Parkinson's disease patients in a clinical trial. (**A**) A manikin head demonstrating the drug delivery system used in the study. An application set coming from infusion pumps was attached to a skull-mounted transcutaneous port. Intracranially implanted catheters were also connected to the skull-mounted port. (**B**) The skull-mounted port was relatively imperceptible. It was the only external component when a patient was not receiving an infusion. (**C**) An axial MRI image at the level of the putamen shows two of catheters entering either side of the brain posteriorly and reaching the putamen. Gadolinium test infusate can be seen distributed throughout both putamen. (**D**) A single patient receiving GDNF infusion. (**E**) Three patients receiving their monthly GDNF infusions in a study infusion suite. Image reproduced from (Whone et al. 2019), under the terms of the Creative Commons Attribution 4.0 International.

2.6.2 Clinical studies with NRTN

Supported by the promising safety and efficacy outcomes in preclinical animal studies, intraparenchymal injections of AAV2 vector encoding human NRTN (CERE-120) have been evaluated in several clinical trials (Table 2.4.). In an open-label phase I study, AAV2-NRTN injected into the putamen showed evidence for safety and produced a 36% increase in the off-medication UPDRS motor score as compared to the baseline at 12 months (Marks et al. 2008). However, in a 12-month double-blind phase II trial, the intraputamenal AAV2-NRTN gene transfer failed to show superiority to sham surgery when assessed using the off-medication UPDRS motor score (Marks et al. 2010). Interestingly, a subgroup of patients, who were followed in a double-blind manner up to 18 months, revealed a small but significant clinical benefit in favor of AAV2-NRTN suggesting a possibility for a delayed effect. In addition, the gene therapy was well tolerated and improved several secondary outcome measures both at 12 and 18 months.

In contrast to the findings in MPTP-treated monkeys (Kordower et al. 2006; Herzog et al. 2007), brain autopsy data from four PD subjects who died from unrelated causes after intraputamenal administration of AAV2-NRTN showed impaired axonal transport of NRTN from the putamen to SNpc (Bartus et al. 2011; Bartus et al. 2015). To overcome this axonal transport deficiency, the next studies utilized a new dosing paradigm: AAV2-NRTN was injected into the SNpc in combination with putamenal delivery to activate repair genes directly in the cell bodies, and thereby enhance the potential trophic effects. Nonetheless, after encouraging safety outcomes in an open-label phase I study (Bartus et al. 2013), a sham-surgery controlled double-blind phase II trial failed to meet the primary endpoint assessed by the off-state UPDRS motor score at 15-month time point (Olanow et al. 2015). *Postmortem* analysis of two patients from these studies demonstrated that AAV2-NRTN delivery provided persistent, but spatially restricted, transgene expression in the putamen and SN still at 8 and 10 years after the transfection (Chu et al. 2020). NRTN expression was associated with focal sprouting and upregulation of TH expression, but not with antiparkinsonian effects such as reduced Lewy pathology when compared to untreated PD control subjects.

2.6.3 The first clinical study with CDNF

The first-in-human, double-blind, placebo-controlled phase I/II study investigating the safety and efficacy of intraputamenal CDNF application in 17 patients with moderate PD was recently completed (Huttunen and Saarma 2019; ClinicalTrials.gov identifier NCT03295786). The study consisted of two parts: the initial 6-month main study in which patients were assigned to receive either placebo or two dose levels of CDNF, and the 6-month open-label extension part during which all patients received one of the two doses of CDNF. The company announced in a press release that the study achieved its primary endpoint of safety and tolerability at 12 months (Herantis Pharma Plc, press release 27 August 2020). Two patients had to discontinue the study due to adverse events related to the implanted dose delivery system. Treatment-related adverse events, however, were transient and mild. The preliminary non-statistical assessment of the secondary endpoints suggests an improvement in the UPDRS motor score in some patients at 12 months supporting the potential efficacy of CDNF therapy. The exploratory endpoints included DAT-PET imaging which showed encouraging response in the putamen of some CDNF-treated individuals. Noteworthily, the final analyses of the secondary and exploratory outcomes including CSF proteomics and α-syn measurements are still underway.

3 AIMS OF THE STUDY

The promising preclinical results of GDNF and NRTN have failed to translate into an effective disease-modifying treatment for PD. Therefore, to fulfill this unmet medical need, new approaches for neurotrophic therapies have to be explored. The studies were executed to support the preclinical characterization of unconventional NTFs, CDNF and MANF, and novel small molecule RET agonists. We aimed to uncover the functional effects of intracranially administered CDNF and MANF in the normal rat brain and to clarify the neuroprotective potential of RET agonists in a rat model of PD.

The specific objectives were to determine:

1. the effects of exogenously administered GDNF, CDNF and MANF on dopamine and GABA release and dopamine synthetizing and metabolizing enzymes in the normal rat brain (Study I)

2. the diffusion and transport properties of CDNF after injection to the substantia nigra of non-lesioned rats (Study II)

3. the neuroprotective and neurorestorative effects of small molecule RET agonists in a 6-OHDA model of PD in rats (Studies III and IV).

4 MATERIALS AND METHODS

The main methods used by the author in studies I-IV are shortly described here. More comprehensive descriptions of the materials and methods can be found in the original publications and their supplementary materials which are attached to the thesis.

4.1 Animals

Adult Wistar male rats (RccHan:WIST, Harlan, the Netherlands) were used for all experiments in study I and II, for the neuroprotection experiment in study III and for the neurorestoration experiment in study IV. Rats were normally housed in groups of 3-4 per cage under a 12h light/dark cycle (lights on 06:00-18:00). All experiments were performed during the light period. Rats had *ad libitum* access to rat chow and tap water at all times. In study I, rats were moved to individual microdialysis cages (30 × 30 × 30 cm) after the surgery. In studies III and IV, rats were housed in individual cages during the brain infusion of the treatments but regrouped again after removing the infusion cannulas and minipumps. The animal experiments were reviewed and approved by the National Animal Experiment Board of Finland (ESLH-2009-05234/Ym-23; ESAVI/5459/04.10.03/2011; ESAVI/7551/04.10.07/2013; ESAVI/ 198/04.10.07/2014 and ESAVI/7812/04.10.07/2015) and carried out in accordance with the European Union directive 2010/63/EU on the protection of animals used for scientific purposes.

4.2 Drugs, toxins and neurotrophic therapies

4.2.1 Neurotrophic factors

The following NTFs were used: recombinant human (rh) GDNF (ProSpec-Tany TechnoGene Ltd., Israel) (study I and unpublished experiment), rhGDNF (PeproTech Inc., USA) (studies III and IV), rhCDNF (Biovian Oy, Finland) (studies I, II and unpublished experiment) and rhMANF (Icosagen AS, Estonia) (study I and unpublished experiment). NTFs were diluted in sterile phosphate-buffered saline, pH 7.4 (PBS) which also served as a vehicle control in studies I, IV and unpublished experiment.

4.2.2 Small molecule RET agonists

BT13 (N,N-diethyl-3-(4-(4-fluoro-2-(trifluoromethyl)benzoyl)piperazin-1-yl)-4-methoxy-benzene-sulfonamide), MW 517.54 g/mol (study III), and BT44 ((4-5-((3,4-dihydroisoquinolin-2(1H)-yl)sulfonyl)-2-methoxy-phenyl)piperazin-1-yl(4-fluoro-2-(trifluoromethyl)phenyl)-methanone), MW 577.59 g/mol (study IV), were synthesized by EvoBlocks Ltd. (Hungary). The structure and integrity of the compounds were verified by NMR spectrometry, and the purity by liquid chromatography–mass spectrometry (BT13, 98.6%) or high-performance liquid chromatography (HPLC) (BT44, 97.3%). BT13 and BT44 were dissolved in 100% propylene glycol (PG) for the *in vivo* neuroprotection and -restoration experiments due to limited solubility in aqueous solutions

(studies III and IV). Their long-term stability in PG at 37°C was confirmed before the *in vivo* experiments. PG was used as a vehicle control in the studies.

4.2.3 Neurotoxin

In studies III and IV, dopaminergic lesions were induced using intracranial injections of 6-OHDA (6-hydroxydopamine hydrochloride, Sigma-Aldrich GmbH, Germany), dissolved in ice-cold, de-oxygenated saline with 0.02% ascorbic acid. Desipramine hydrochloride 15 mg/kg, i.p. (calculated as free base; Sigma-Aldrich) was administered 30 min before the 6-OHDA injection to prevent the uptake of 6-OHDA into noradrenergic nerve terminals.

4.2.4 Drugs

Isoflurane (Vetflurane® 1000 mg/g, Virbac SA, France) was administered via inhalation to induce and maintain general anesthesia during stereotaxic surgeries. Lidocaine-adrenalin solution (Lidocain 10 mg/ml c. adrenalin, Orion Pharma Oyj, Finland) was used for local anesthesia. To relieve postoperative pain, rats received tramadol 1 mg/kg, s.c. (Tramal® 50 mg/ml, Orion Pharma) at the end of the surgeries (study I), or buprenorphine 0.05 mg/kg, s.c. (Temgesic® 0.3 mg/ml, Indivior UK Limited, United Kingdom) before and carprofen 5 mg/kg, s.c. (Rimadyl Vet® 50mg/ml, Zoetis Inc., USA) immediately after the surgeries (studies II-IV). Additional doses of the analgesics were given one day later.

D-amphetamine sulfate (Division of Pharmaceutical Chemistry and Technology, University of Helsinki, Finland) was used to stimulate dopamine overflow from striatal nerve terminals in study I and induce turning behavior in unilaterally 6-OHDA lesioned rats in studies III and IV. In GABA microdialysis experiment (unpublished), nipecotic acid (Sigma-Aldrich) served as a universal inhibitor of GABA transporters (IC50 ~18 µM). For *in vivo* TH activity experiment in study I, rats were administered with 3-hydroxybenzylhydrazine (NSD1015) (Sigma-Aldrich) to inhibit AADC in the brain. Clorgyline (IC50 ~1.2 nM) and pargyline (IC50 ~8.2 nM) (included in the Monoamine Oxidase Assay Kit, MAK136, Sigma-Aldrich) were used to irreversibly inhibit MAO-A and MAO-B activities, respectively, in MAO activity experiment in study I. Before transcardial perfusions in studies II-IV, rats were deeply anesthetized with sodium pentobarbital (Mebunat Vet® 60 mg/ml, Orion Pharma).

4.3 Stereotaxic surgeries

All stereotaxic surgeries were performed under isoflurane anesthesia (3.5–4.5% during induction and 2.0–3.5% during maintenance) using a stereotaxic frame (Stoelting Co., USA). The skull was exposed and burr holes were made using a high-speed drill (Foredom Electric Co., USA). NTFs, RET agonists, vehicles and 6-OHDA were administered into the target brain regions as described below. Brain injections were made using an electronic microinjector (Quintessential stereotactic injector, Stoelting) and 10-µl syringes with 26G (Hamilton Co., USA) (studies I-III) or 33G (NanoFil, World Precision Instruments, USA) (study IV) blunt tapered needle attached. At the completion of the injections, the needle was kept in place for 4-5 min to minimize backflow of the solution. All

coordinates were determined relative to the bregma, according to the rat brain atlas (Paxinos and Watson 1998). After the surgeries, rats were allowed to recover at least one week before microdialysis or behavioral experiments.

4.3.1 Neurotrophic factor injections and guide cannula implantation

In study I, a unilateral injection of GDNF, CDNF or MANF (10 µg in 5 µl) or PBS (5 µl) was made into the left dorsal striatum (A/P +1.0, M/L +2.7, D/V −5.0 mm). The injection rate was 1.0 µl/min. For the enzyme activity assays, the incision was sutured after the injection. For the microdialysis experiments, a guide cannula (BASi MD-2250, Bioanalytical Systems Inc., USA) was implanted after the NTF injection. The tip of the cannula was placed into the left dorsal striatum (A/P +1.0, M/L +2.7, D/V − 4.0 mm) for dopamine release measurements, or into the left GPe (A/P -1.4, M/L +3.5, D/V -5.5 mm) for GABA release measurements. Thereafter, the cannula was fixed to the skull with three stainless steel screws and polycarboxylate cement (Aqualox, Voco GmbH, Germany). In study II, CDNF (3 µg in 4 µl, or 3 µg in 1 µl) was injected into the left SN (A/P −5.4, M/L +2.0, D/V −7.2 mm) with the flow rate of 0.5 µl/min. The incision was sutured after the injection.

4.3.2 6-OHDA injections

In study III, rats received a single unilateral injection of 6-OHDA (16 µg in 4 µl; calculated as free base) into the left dorsal striatum (A/P +1.0, M/L +2.7, D/V -4.0 mm) at the flow rate of 1.0 µl/min. In study IV, 6-OHDA injections were made into three different sites in the right dorsal striatum (A/P +1.6, M/L -2.8, D/V -6.0 mm; A/P 0.0, M/L -4.1, D/V -5.5 mm; and A/P -1.2, M/L -4.5, D/V -5.5 mm) with the dose of 3 µg in 1.5 µl per site and flow rate of 0.5 µl/min (Penttinen et al. 2016). The needle was kept at a 10° angle to avoid lateral ventricles. Lastly, the scalp incision was closed with sutures.

4.3.3 Infusions of RET agonists and GDNF with osmotic minipumps

Osmotic infusion pumps (Alzet Osmotic pump model 2002, Durect Co., USA), connected with catheter tubing to brain cannulas (Alzet Brain infusion kit no. 2, Durect), were used to deliver RET agonists, GDNF and vehicles into the lesioned dorsal striatum in studies III and IV. The pumps were aseptically filled with study solutions and allowed to reach the steady-state pumping rate by incubating overnight in isotonic saline at 37°C. The pumps and cannulas were implanted in a stereotaxic surgery one hour (study III) or two weeks (study IV) after the 6-OHDA injections. The pump was placed into a subcutaneous pocket between the scapulae. The tip of the cannula was lowered to coordinates A/P +1.0, M/L +2.7, D/V -4.0 mm (study III) or A/P +0.2, M/L -3.0, D/V -5.0 mm (study IV) after which the cannula was secured to the skull with three stainless steel screws and polycarboxylate cement (Aqualox).

In study III, the pumps constantly delivered BT13 ~3-6 µg/24h (0.25-0.5 µg/µl), GDNF 3 µg/24h (0.25 µg/µl) or 100% PG into the dorsal striatum at the flow rate of 0.5 µl/h for seven days. The dose for BT13 could not be determined accurately because the solubility of BT13 in 100% PG was limited to 0.5 µg/µl in stabile test tube conditions at 37°C. In study IV, the pumps infused BT44 0.1

µg/24h (8.3 ng/µl), BT44 0.3 µg/24h (25 ng/µl), GDNF 3 µg/24h (0.25 µg/µl), 100% PG or PBS at the flow rate of 0.5 µl/h for 14 days. The doses for BT44 were selected based on the potency of the compound in neuronal survival assay *in vitro* where it showed at least 10 times higher potency as compared to BT13. Therefore, 10-20 and 30-60-times lower doses of BT44 were chosen for study IV than were used for BT13 in study III. After the treatment infusions, the pumps, cannulas and screws were removed, and the incision was cleaned, disinfected and sutured.

4.4 Microdialysis experiments

Microdialysis experiments were carried out in freely-moving rats one and three weeks after the stereotaxic injection of NTFs and implantation of guide cannulas (study I and unpublished experiment). The one-week time point was selected based on an earlier brain microdialysis experiment with GDNF in freely-moving rats (Xu and Dluzen 2000). The three-week time point served to probe the long-term effects of NTFs on dopamine and GABA transmission. Proper function of the microdialysis probes (BASi MD-2200, Bioanalytical Systems, membrane length 2 mm) was verified with *in vitro* recovery test at room temperature before the experiments but *in vivo* dialysate concentrations were not corrected for *in vitro* recoveries. In each experiment, the probe was inserted into the guide cannula and perfusion of the microdialysis membrane was started with modified Ringer solution (147 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.04 mM ascorbic acid) at the flow rate of 2.0 µl/min. After a two-hour stabilization period, dialysate samples were collected every 15 min for 270 min (study I) or 150 min (unpublished experiment) as described below and in Figure 4.1. To minimize variation between the subjects, the results were analyzed as percent changes of the analyte concentrations from the baseline levels instead of actual analyte concentrations. In addition, the conventional microdialysis (as opposed to no-net-flux microdialysis) used here is not a suitable method to measure exact analyte concentrations in the extracellular fluid. After the first microdialysis experiment at week 1, the probe was removed from the brain. After the second experiment at week 3, rats were sacrificed and the brains were collected to verify the correct placements of the probes. Data only from the rats with accurate probe placements were included in the analyses.

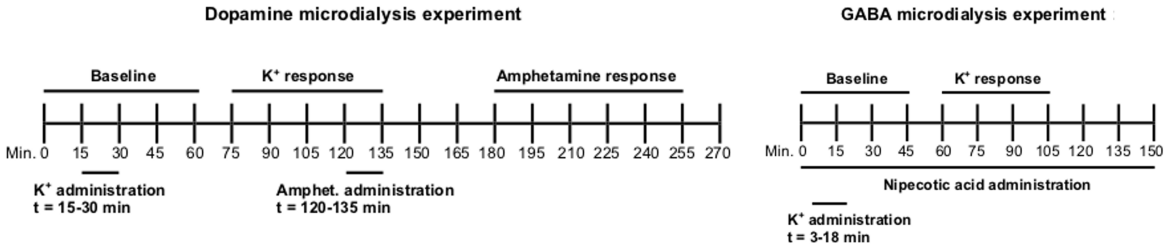

Figure 4.1. Time course of the dopamine and GABA microdialysis experiments. The lag time between the administration of a stimulation solution and the corresponding response is due to slow flow rate of the perfusion solution (2.0 µl/min) and large dead volume of the long tubes. Figure drawn by the author.

In the dopamine microdialysis experiment (study I), dopamine, DOPAC and HVA concentrations in the first four samples were used to calculate the baselines. To depolarize the striatal nerve terminals and evoke dopamine release, 100 mM potassium solution (27.5 mM NaCl, 100 mM KCl,

1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.04 mM ascorbic acid) was administered for 15 min via reverse dialysis starting at 15 min. To stimulate dopamine release for the second time by draining synaptic dopamine vesicles and reversing DAT function, 100 µM D-amphetamine (in modified Ringer) was administered via reverse dialysis for 15 min starting at 120 min. The analyte concentrations were quantified right after collecting the samples using a HPLC system with a C18 reverse-phase column (Kinetex C18, Phenomenex Inc., USA) and an electrochemical detector (Coulochem II, ESA Biosciences Inc., USA).

In the GABA microdialysis experiment (unpublished), reuptake of GABA into neurons and glial cells was blocked with 100 µM nipecotic acid which was included in the Ringer solution during the whole experiment (Vihavainen et al. 2008). The baseline level for GABA was calculated as an average in the first three samples. To stimulate GABA release, modified Ringer solution with 50 mM potassium (100 µM nipecotic acid, 99.7 mM NaCl, 50 mM KCl, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$) was delivered for 15 min starting at 3 min (reverse dialysis). The samples were stored at -80°C until analyzing with a HPLC system equipped with a C18 reverse-phase column (Kinetex C18, Phenomenex) and a fluorescence detector (Jasco FP-1520, Jasco Co., United Kingdom) as described in detail elsewhere (Julku et al. 2016; Piepponen and Skujins 2001). To enable sensitive fluorometric detection of GABA, it was derivatized with a mixed *o*-phthaldialdehyde (Sigma-Aldrich) and 2-mercaptoethanol (Sigma-Aldrich) reagent before the samples were injected into the column.

4.5 Enzymes activity measurements

Seven days after the intrastriatal injections of NTFs or vehicle in study I, tissue samples were collected from the dorsal striatum for enzyme activity measurements. Rats were decapitated, and the brains were excised rapidly and rinsed in ice-cold saline. Bilateral striatal punches were collected from coronal slices with a 3-mm sample corer, snap frozen on dry ice and stored at -80°C. Later, the samples were weighed, homogenized and centrifuged.

For *in vivo* TH activity measurement, rats were administered with a BBB penetrating AADC inhibitor NSD1015 100 mg/kg, i.p., 30 min before tissue collection (Vihavainen et al. 2008). After sample preparation, the S1 supernatants filtered with Vivaspin® 500 filter concentrators (10,000 MWCO PES; Sartorius Stedim Biotech GmbH, Germany) were analyzed with a HPLC system equipped with a C18 reverse-phase column (Kinetex XD-C18, Phenomenex) and an electrochemical detector (ESA CoulArray Electrode Array, ESA Biosciences) to measure the amount of accumulated L-DOPA as ng/g wet weight of the tissue specimen (Valros et al. 2015).

For COMT, MAO-A and MAO-B activity assays, the total protein concentration was determined using bicinchoninic acid method (Pierce™ BCA Protein Assay Kit, ThermoFisher Scientific Inc., USA). Total COMT activity was measured as described in (Schendzielorz et al. 2012; Smith et al. 2014). The reaction products, vanillic and isovanillic acid, were analyzed with HPLC using an electrochemical detector (Coulochem II, ESA Biosciences). MAO-A and MAO-B activities were determined with Monoamine Oxidase Assay Kit (MAK136, Sigma-Aldrich). To differentiate MAO-A and MAO-B activities, isoform-specific inhibitors (5 µM of clorgyline for MAO-A and 5 µM of

pargyline for MAO-B) were used. The fluorescence of the samples was measured with a multi-well plate reader (Victor2, 1420 Multilabel Plate Reader, PerkinElmer Inc., USA) using wavelengths 530 nm and 590 nm for excitation and detection, respectively.

4.6 Behavioral tests

In studies III and IV, behavioral tests were conducted at defined timepoints as indicated in Figure 4.2. which shows the designs of the neuroprotection (A; study III) and neurorestoration (B; study IV) experiments. All behavioral assessments were carried out in a blinded manner.

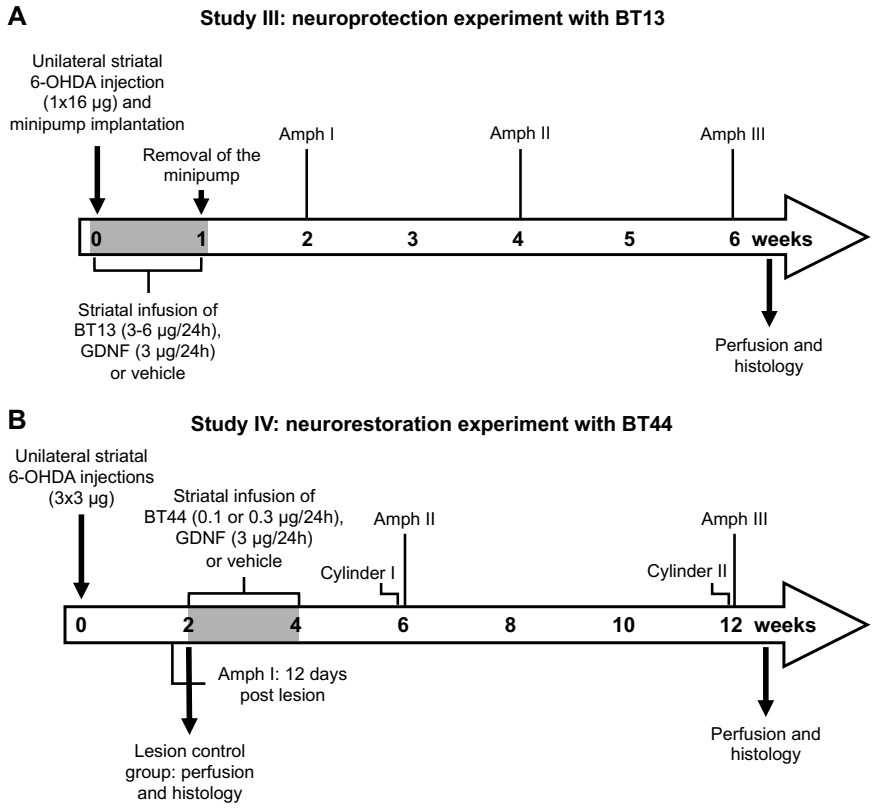

Figure 4.2. Experimental design for the neuroprotection and neurorestoration experiments. The neuroprotective effect of BT13 (**A**) and neurorestorative effect of BT44 (**B**) were investigated in a unilateral 6-OHDA model of PD in rats. Amph I-III: amphetamine-induced (2.5 mg/kg, i.p.) rotational asymmetry tests, Cylinder I and II: cylinder tests. Figure drawn by the author.

4.6.1 Amphetamine-induced rotational behavior

Amphetamine-induced rotational asymmetry test was used to measure motor deficits arising from the unilateral 6-OHDA lesion of the nigrostriatal dopamine system. Rotational behavior was monitored in automated rotometer bowls (Med Associates Inc., USA) as described previously in (Ungerstedt and Arbuthnott 1970; Lindholm et al. 2007). After a 30-min habituation period, rats received a single injection of D-amphetamine 2.5mg/kg, i.p. (calculated as free base). The number of full (360°) uninterrupted clockwise and counterclockwise turns was recorded for 120 min. Net

ipsilateral turns to the lesion side were calculated by subtracting contralateral turns from ipsilateral turns.

4.6.2 Cylinder test

In study IV, cylinder test was used to evaluate spontaneous limb-use asymmetry. Cylinder tests were carried out before the amphetamine-induced rotational asymmetry tests using a protocol described by (Schallert et al. 2000; Tillerson et al. 2001). Briefly, rats were placed in a transparent plexiglass cylinder (diameter ~20 cm, height ~30 cm) and their exploratory activity was recorded for 10 min with a video camera placed under the cylinder. The number of weight-shifting movements against the wall was scored individually for the ipsilateral (non-impaired) and contralateral (impaired) forepaws. A simultaneous placement of both forepaws on the wall as well as lateral exploration along the wall by alternating right and left forepaw placements were scored as “both paw contacts”. After a rear, the first weight-receiving contact to the ground was scored as a landing contact for the forepaw used for landing, or as a “both paw contact” if both paws contacted the ground simultaneously. Percentual limb-use asymmetry score was first calculated separately for wall exploration and landing using the following formula (Schallert and Woodlee 2005):

$$\frac{ipsilateral\ contacts + 0.5 \times\ both\ paw\ contacts}{ipsilateral + contralateral + both\ paw\ contacts} \times 100\%$$

Finally, the mean of these scores was used as an averaged asymmetry score. Scores >50% indicate greater reliance on the ipsilateral (non-impaired) forelimb and <50% on the contralateral (impaired) forelimb.

4.7 Perfusion and brain sectioning

After completing the microdialysis (study I) or behavioral experiments (studies III and IV), or 2-24 h after CDNF injections in the diffusion study (study II), rats were deeply anesthetized with sodium pentobarbital 90 mg/kg, i.p., and transcardially perfused with PBS and 4% paraformaldehyde (PFA) in PBS. The brains were removed, post-fixed in 4% PFA overnight and stored in 20% sucrose in PBS at 4°C until snap freezing in dry ice-cooled isopentane. The frozen brains were cut into 40 µm-thick coronal sections with a cryostat (Leica CM3050, Leica Biosystems, Germany). The sections were collected in a series of six sections and stored in cryoprotective buffer at -20°C. For the diffusion study (study II), some brains were embedded in paraffin and cut to 5 µm sagittal sections (M/L 1.4-3.4 mm), taking every 10th section. The paraffin sections were collected on microscope slides and stored at 4°C.

4.8 Immunohistochemistry

Immunohistochemical labelling was performed on free-floating coronal (studies II-IV) or paraffin-embedded sagittal (study II) sections using standard immunohistochemical procedures as described in detail in the original publications. The primary and secondary antibodies used in the experiments are listed in Table 4.1.

Table 4.1. Primary and secondary antibodies used for immunohistochemical labelling.

Primary antibodies				Secondary antibodies				Studies
Antibody	**Cat#, Supplier**	**Reactivity**	**Concentration; Incubation**	**Antibody**	**Conjugation**	**Cat#, Supplier**	**Concentration; Incubation**	
Rabbit polyclonal anti-hCDNF	300-100, Icosagen	Human, mouse	Free-floating: 1:500; +4°C o/n	Goat anti-rabbit	Biotin	BA-1000, Vector Laboratories Inc., USA	1:200; RT 1h	II
			Paraffin: 1:1000; +4°C o/n	Horse anti-rabbit	Biotin	BA-1100, Vector Laboratories	1:200; RT 1h	II
				Goat anti-rabbit	AlexaFluor488	A-11034, ThermoFischer Scientific	1:200; RT 2h	II
Mouse monoclonal anti-parvalbumin	MAB1572, Merck Millipore KGaA, Germany	Rat, mouse, rabbit, human etc.	Paraffin: 1:1000; +4°C o/n	Goat anti-mouse	AlexaFluor568	A-11004, ThermoFischer Scientific	1:200; RT 2h	II
Mouse monoclonal anti-TH	MAB318, Merck Millipore	Rat, mouse, human, zebrafish etc.	Free-floating: 1:2000; RT o/n	Horse anti-mouse	Biotin	BA-2001, Vector Laboratories	1:200; RT 2h	III
			Paraffin: 1:500; +4°C o/n	Goat anti-mouse	AlexaFluor568	A-11004, ThermoFischer Scientific	1:200; RT 2h	II
Rat monoclonal anti-DAT	MAB369, Merck Millipore	Rat, mouse, human, monkey	Free-floating: 1:2000; +4°C o/n	Rabbit anti-rat	Biotin	BA-4000, Vector Laboratories	1:200; RT 1h	III
Rabbit polyclonal anti-TH	AB152, Merck Millipore	Rat, mouse, human, fruit fly etc.	Free-floating: 1:2000; RT o/n	Protein A (binds to IgG:s of rabbit, mouse, human, guinea pig etc.)	Biotin	MP Biomedicals, USA (protein A), Sigma-Aldrich (N-hydroxysuccinimido-biotin), conjugated in-house	1:100; RT 1h	IV
Rabbit monoclonal anti-DAT	ab184451, Abcam Plc., United Kingdom	Rat, mouse	Free-floating: 1:500; +4°C o/n	Goat anti-rabbit	Biotin	BA-1000, Vector Laboratories	1:500; RT 1h	IV

RT, room temperature; o/n, overnight; TH, tyrosine hydroxylase; DAT, dopamine transporter; IgG, immunoglobulin G

4.9 Histological analyses

The striatum and midbrain sections were stained for TH and DAT to assess the density of dopaminergic fibers in the striatum and the number of dopamine neurons in the SNpc.

4.9.1 Fiber density in the striatum

The optical densities of TH-ir and DAT-ir fibers in the dorsal striatum were measured bilaterally from three different rostro-caudal levels through the striatum from each rat (studies III and IV). The analyses were performed under blinded conditions. First, digital images of the immunostained sections were acquired with an automated bright field microscope whole slide scanner Pannoramic P250 Flash II (3DHistech Ltd., Hungary). The images were converted to 8-bit gray scale, and colors were inverted. The dorsal striata were outlined, and integrated optical densities divided by the size of the outlined areas were measured with Fiji ImageJ software (Media Cybernetics Inc., USA). All density values were corrected for nonspecific background staining measured from the corpus callosum. The data are expressed as percentage of the fiber density on the lesioned hemisphere as compared to the intact hemisphere. The total magnitude of striatal denervation was estimated as an average reduction in the fiber density at the three levels measured.

4.9.2 Cell counts in the SNpc

In studies III and IV, the number TH-ir cells in the SNpc was estimated in a blinded manner with the optical fractionator method and dissector principle according to unbiased counting rules (West et al. 1991). Olympus BX51 microscope (Olympus Co., Japan) was connected to a computer running Stereo Investigator platform (MBF Bioscience Inc., USA), and cell counting was done bilaterally under 60x magnification (Olympus PlanApo 60x 1.40 Oil /0.17 objective) from three sections per brain at approximately the same rostro-caudal levels as described earlier (Voutilainen et al. 2009). The results are presented as percentage of cells in the lesioned SNpc as compared to the intact SNpc.

Additionally, in study IV, TH-ir cells bodies in the SNpc were counted using an automated deep convolutional neural networks (CNN) algorithm in a cloud-embedded image processing platform (Aiforia Technologies Oy, Finland). This computer-assisted cell counting method, that is based on supervised machine learning and automated image recognition, is described in detail and validated in (Penttinen et al. 2018). Briefly, digitized images of TH-immunolabelled sections were uploaded to Aiforia^{TM} platform. The SNpc was demarcated bilaterally in six sections from each brain. CNN algorithm, that was trained to recognize dopaminergic cell bodies from digital images, counted the number of TH-ir neurons within the demarcated areas. The number of neurons was summed up from the six sections separately for both hemispheres. The performance of the algorithm was confirmed against stereological assessment of TH-ir cell counts in 20 randomly selected brains in study IV. The results obtained with CNN algorithm and stereological counting method had a strong positive correlation (Pearson's $r = 0.83$, $p < 0.001$).

4.10 Statistical analyses

All results are presented as mean ± standard error of the mean (SEM). The statistical analyses were conducted with SPSS® Statistics 24 software (IBM SPSS Inc., USA). The group sizes and NTF doses were selected on the basis of our earlier experience (Airavaara et al. 2006; Käenmäki et al. 2010; Lindholm et al. 2007; Voutilainen et al. 2011, 2009). Paired two-tailed Student's *t*-test was used for two-sample within-subject comparisons and unpaired two-tailed Student's t-tests for two-sample between-subject comparisons. Group-wise analyses were done using one-way analysis of variances (ANOVA) in case of between-group comparisons, or repeated measures ANOVA in case of within-group comparisons, followed by Ryan-Einot-Gabriel-Welsch F (REGWF; study I) or Tukey HSD (studies II-IV) *post hoc* tests. To assess the strength of a linear association between two variables, Pearson correlation test was applied. The results were considered to be statistically significant at $p < 0.05$.

5 RESULTS

5.1 Injection of neurotrophic factors into the intact rat brain

5.1.1 Effects of exogenous neurotrophic factors on dopamine release and turnover in the striatum (I)

In the microdialysis experiment one week after the intrastriatal NTF injections, the baseline levels of extracellular dopamine, DOPAC or HVA did not differ between the treatment groups. Both potassium and amphetamine-evoked release of dopamine was significantly elevated in MANF-injected rats as compared to vehicle or GDNF-injected rats (Figure 5.1.A and B). There was also a trend for increased stimulus-evoked dopamine release in CDNF-injected rats, but this effect did not reach statistical significance when compared to vehicle. At three weeks post injection, there were no more significant differences in dopamine release between the treatment groups (Study I, supplementary material).

Reflecting enhanced dopamine metabolism, MANF induced a significant increase in dopamine turnover in the striatum as evaluated by calculating DOPAC/dopamine ratio in the microdialysis samples at one week, but not anymore at three weeks post injection (Figure 5.1.C). There were no significant differences in HVA/dopamine ratio between the treatment groups at one or three weeks after the injection.

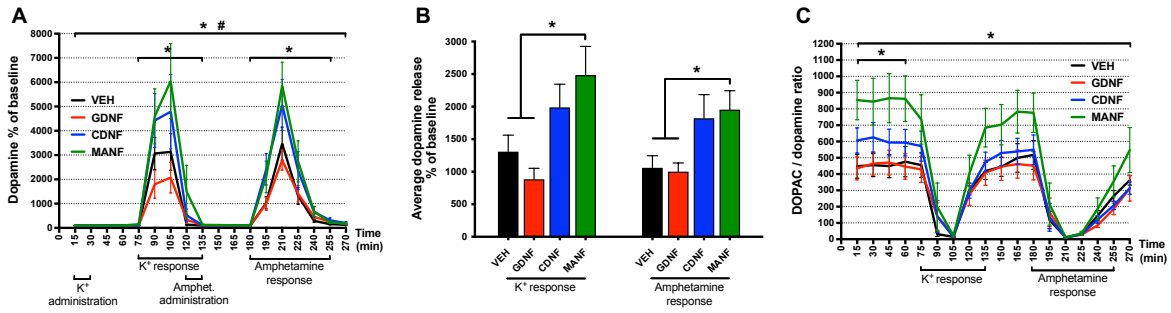

Figure 5.1. Striatal dopamine release and turnover measured with brain microdialysis one week after an intrastriatal injection of neurotrophic factors. (**A**) Potassium- and amphetamine-evoked (75–135 min and 180–255 min, respectively) dopamine release was increased in MANF group as compared to vehicle and GDNF groups (*p < 0.05; REGWF after repeated measures ANOVA; 75–135 min: $F_{3,36}$ = 4.874, p = 0.006; 180–255 min: $F_{3,36}$ = 3.683, p = 0.021). In addition, the overall (15–270 min) extracellular concentration of dopamine was elevated in MANF group as compared to vehicle and GDNF groups (*p < 0.05) and in CDNF group as compared to GDNF group (#p < 0.05; REGWF after repeated measures ANOVA; 15–270 min: $F_{3,36}$ = 4.678, p = 0.007). (**B**) Averaged total potassium- and amphetamine-evoked dopamine overflow was augmented in MANF group when compared to vehicle and GDNF groups (*p < 0.05; REGWF after one-way ANOVA; K^+ response: $F_{3,36}$ = 4.874, p = 0.006; Amphetamine response: $F_{3,36}$ = 3.683, p = 0.021). (**C**) DOPAC/dopamine ratio was higher in MANF-injected rats as compared to vehicle and GDNF-injected rats during the whole experiment and in the baseline samples (*p < 0.05; REGWF after repeated measures ANOVA; 15–270 min: $F_{3,36}$ = 3.065, p = 0.040; 15–60 min: $F_{3,36}$ = 3.868, p = 0.017). The dose of each NTF was 10 µg. Results are shown as % of the baseline (A and B) or concentration ratio (C); mean ± SEM; n = 10. Figures adapted from (Renko et al. 2018), under the terms of the Creative Commons Attribution 4.0 International.

5.1.2 Effects of exogenous neurotrophic factors on dopamine neurochemistry (I)

The amount of L-DOPA, the precursor of dopamine, accumulated into striatal tissue samples after AADC inhibition by NSD1015 provides a direct measure for L-DOPA production rate indicating the *in vivo* TH activity of the nigrostriatal pathway (Carlsson et al. 1972). At one week post NTF injection, TH activity was increased approximately by 60% in the dorsal striatum of rats administered with GDNF as compared with the vehicle-administered controls (Figure 5.2.A). MANF also tended to increase TH activity (approximately by 50%), but the effect was not statistically significant.

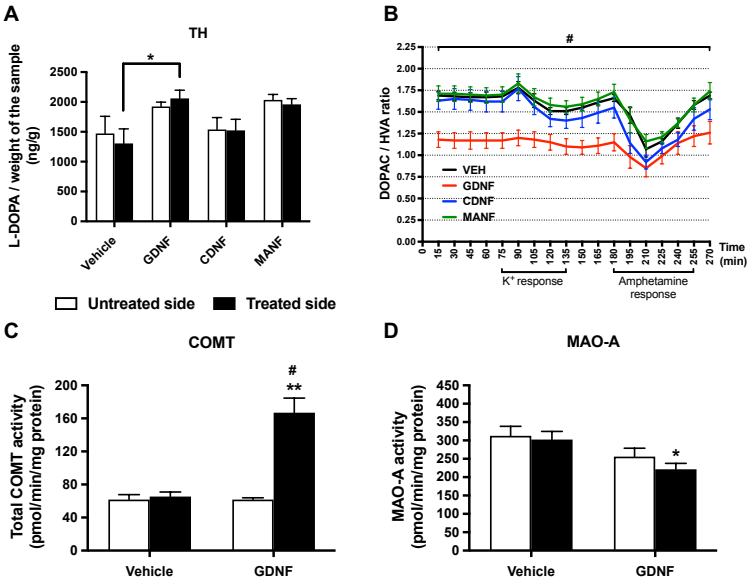

Figure 5.2. Effects of neurotrophic factors on dopamine neurochemistry-regulating enzymes one week after an intrastriatal injection. (**A**) *In vivo* TH activity was increased in GDNF-injected rats when compared with vehicle-injected rats as analyzed by measuring the amount of accumulated L-DOPA in the striatal samples after AADC inhibition with NSD1015 (*p < 0.05; REGWF after one-way ANOVA; $F_{3,22}$ = 3.780, p = 0.025). (**B**) DOPAC/HVA ratio was significantly smaller in GDNF group than in the other groups during the whole experiment (#p < 0.05; REGWF after repeated measures ANOVA; 15–270 min: $F_{3,36}$ = 7.397, p = 0.001). (**C**) GDNF injection increased total COMT activity in the striatum when compared to the vehicle-injected striatum (**p < 0.001; unpaired two-tailed Student's *t*-test t(13) = −5.159) as well as compared to the non-injected striatum (#p < 0.001; paired two-tailed Student's *t*-test t(7) = 6.041). (**D**) MAO-A activity in the GDNF-injected striatum was reduced as compared to the vehicle-injected striatum (*p = 0.011; unpaired two-tailed Student's *t*-test t(14) = 2.944). The dose of the NTFs was 10 µg. Mean ± SEM; n = 6-7 (A), n = 10 (B), n = 7-8 (C, D). Figures adapted from (Renko et al. 2018), under the terms of the Creative Commons Attribution 4.0 International.

Interestingly, the concentration of dopamine metabolites in the striatal microdialysis samples collected at one week post NTF injections showed that DOPAC/HVA ratio was significantly reduced in rats injected with GDNF as compared to all other groups (Figure 5.2.B). This suggests that GDNF injection modulates the activity of dopamine metabolizing enzymes. Indeed, GDNF significantly increased the total COMT activity by 155% in the injected striatum as compared to vehicle-injected striatum and by 170% when compared to the non-injected striatum (Figure 5.2.C). GDNF also significantly reduced the activity of MAO-A, the isoform primarily responsible for the oxidation of

dopamine in rats (Meiser et al. 2013), in the injected striatum by 27% as compared to the vehicle-injected controls (Figure 5.2.D).

5.1.3 Effects of exogenous neurotrophic factors on GABA release in the GPe (unpublished data)

To test whether intrastriatally injected NTFs modulate the GABAergic neurotransmission in the striatopallidal or striatonigral projection pathways, we collected microdialysis samples from the GPe and SNr of freely-moving rats at one and three weeks after the NTF injections. To stimulate GABA release, the MSN nerve terminals were depolarized by administering high concentration of potassium through the microdialysis probe. According to the pilot experiment, the overall extracellular concentration of GABA did not differ significantly between the treatment groups at one or three weeks post injection, nor were there any significant effects on stimulus-evoked GABA output (Figure 5.3.). At three weeks post injection, there seemed to be a tendency toward increased potassium-evoked release of GABA in CDNF-injected animals, but this difference did not reach statistical significance. We also measured the amount of GABA in the GPe tissue samples collected one week after the intrastriatal NTF injections, but no significant differences were found between the groups. GABA release in the SNr could not be reliably quantified because the concentration of GABA in the dialysates was at the limit of quantitation of the HPLC system and sometimes even below it (we used 1 mm long membranes for the SNr microdialysis).

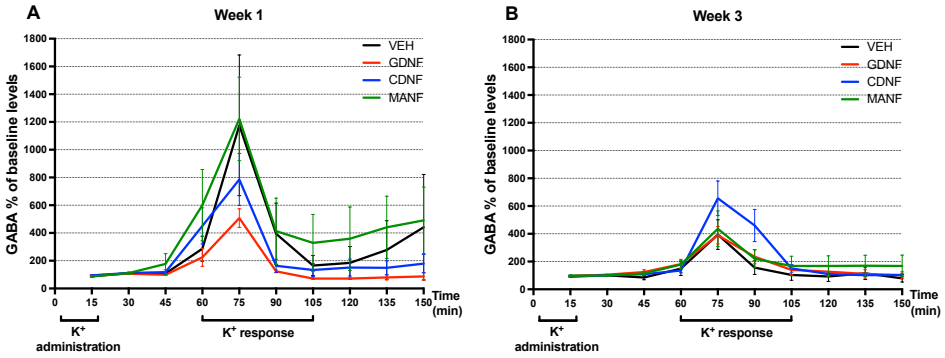

Figure 5.3. GABA release in the GPe measured with brain microdialysis one and three weeks after an intrastriatal injection of neurotrophic factors. There were no significant differences in GABA release between the treatment groups at one week (**A**) or three weeks (**B**) post injection. The dose of each NTF was 10 µg. Results are shown as % of the baseline; mean ± SEM; n = 4-6.

5.1.4 Distribution of CDNF after nigral injection (II)

To characterize the diffusion properties of supra-nigrally injected CDNF (3 µg) in naïve rats, the brains were collected for immunohistochemical analysis at 2, 6 and 24 h after the injection. We observed robust CDNF staining throughout the ipsilateral midbrain including the SNpc at 2 and 6 h post injection, but not anymore at the 24-h time point (Figure 5.4.A-C). Clear CDNF labelling was also detected in the hippocampus and amygdala as well as thalamic and hypothalamic regions. There was no CDNF signal observed in the striatum at any of the time points. Additionally, increasing the injection volume from 1 to 4 µl while keeping all other parameters, such as the amount of CDNF, constant resulted in larger diffusion volume of CDNF around the SNpc at the 2-

h time point as shown in the representative sagittal images of the CDNF staining (Figure 5.4.D). These qualitative data suggest that CDNF readily diffuses to the brain areas surrounding the SN but is eliminated within 24 h after the nigral injection.

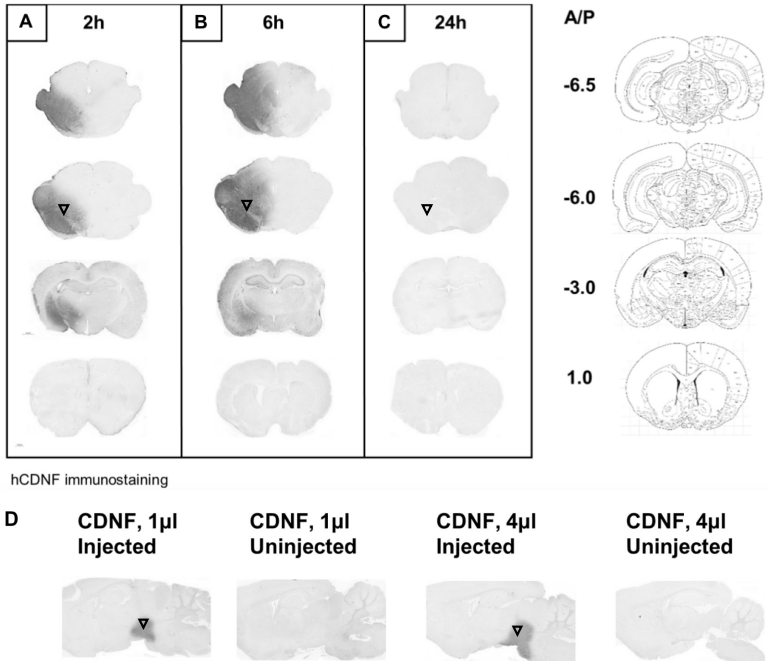

Figure 5.4. Diffusion of human CDNF (hCDNF) after an injection to the substantia nigra (SN) of intact rat brain. (**A-C**) Representative hCDNF immunostaining on coronal sections collected 2, 6 or 24 h after a nigral hCDNF (3 µg/4 µl) injection. CDNF readily diffuses in the brainstem, in particular around the SN, to the hippocampus, amygdala, thalamus and hypothalamus within 2 to 6 h after the injection. The panel on the right shows the rostro-caudal levels of the stained sections. (**D**) Representative hCDNF immunolabelling on sagittal sections collected 2 h after a nigral hCDNF injection in the concentration of 3 µg/1 µl or 3 µg/4 µl. CDNF diffuses farther with the 4-µl injection volume. The arrowheads indicate the approximate injection sites (added afterwards to the original figure); n = 2 at each time point or volume. Figures adapted from (Albert et al. 2019), under the terms of the Creative Commons Attribution 4.0 International.

To further elucidate the localization of CDNF after the nigral injection, the midbrain sections were double stained for CDNF and TH or PV. We observed colocalization of CDNF with TH-ir neurons in the SN, while the colocalization with PV-ir neurons was not evident. Multiple TH-ir cells were detected in the nigral area of each brain, and approximately two thirds of them had CDNF-ir puncta inside their cell body on the injected side (Figure 5.5.). Although these observations are only qualitative, they are in good accordance with the earlier results by (Mätlik et al. 2017).

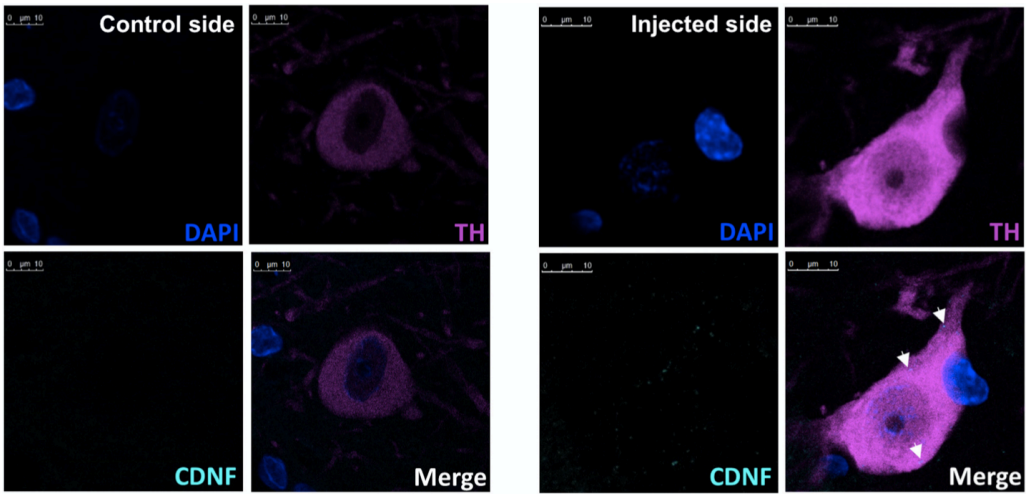

Figure 5.5. Immunofluorescent double staining for human CDNF (hCDNF) and tyrosine hydroxylase (TH) in the substantia nigra (SN). Representative confocal images from a rat brain injected with hCDNF (3 µg) to the SN and perfused 2 h later (right panel). Control side images (left panel) are taken with identical settings to account for possible background staining. White arrows on the injected side indicate hCDNF-ir puncta (cyan dots) within TH-ir cells (magenta) demonstrating the colocalization of hCDNF with dopamine neurons. Scale bars 10 µm; n = 6. Figures adapted from (Albert et al. 2019), under the terms of the Creative Commons Attribution 4.0 International.

When ^{125}I-labeled CDNF was injected to the SN and several brain areas were dissected 24 h later, no radioactivity was detected in distant brain areas such as the striatum, globus pallidus or cortex suggesting the absence of axonal transport to these areas (Figure 5.6.). The highest radioactivity after the nigral injection of ^{125}I-CDNF was measured in the ipsilateral STN. Concurrent administration of either 2000 or 10 000-fold molar excess of unlabeled ("cold") CDNF to the SN together with ^{125}I-CDNF to compete for the active transport mechanisms did not block the spread of ^{125}I-CDNF to the STN.

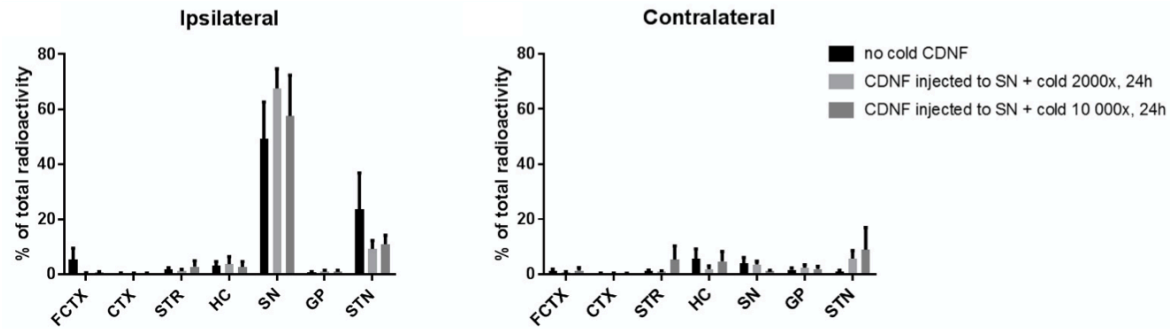

Figure 5.6. Spread of ^{125}I-labeled CDNF after an injection to the substantia nigra (SN) of intact rat brain. Percentage of the total radioactivity (=summed radioactivity in all brain areas measured) in the ipsilateral (left panel) and contralateral (right panel) brain areas measured from tissue samples 24 h after the nigral injection of ^{125}I-CDNF (5 ng). For a subset of animals, 2000 (10 µg) or 10 000-fold (50 µg) molar excess of cold (unlabeled) CDNF was injected together with ^{125}I-CDNF. No transport into the striatum or other brain areas was detected. ^{125}I-CDNF spread into the ipsilateral STN, but this spread was not affected by the excess of cold CDNF. FCTX, frontal cortex; CTX, cortex; STR, striatum; HC, hippocampus; GP, globus pallidus; STN, subthalamic nucleus; mean ± SEM; n = 5-6 per brain region. Figures adapted from (Albert et al. 2019), under the terms of the Creative Commons Attribution 4.0 International.

5.2 Activation of neuronal pro-survival signaling pathways by RET agonists

To facilitate the comparison of pharmacological properties between RET agonists and positive controls (GDNF and NRTN), the molar concentrations of the ligands used in different *in vitro* experiments are provided in Table 5.1. From this comparison it is obvious that the potencies of GDNF and NRTN are at the level of four orders of magnitude larger than those of BT-compounds in the phosphorylation assays, which reflect the increase in protein phosphorylation status after a short-term (15 min) exposure to the ligands. In the neuronal survival and protection assays in cultured dopamine neurons, the potency differences are at the level of one to three orders of magnitude, because the integrated effect of prolonged (5 days) RET activation allowed to use much lower ligand concentrations. Direct potency and efficacy comparisons based on these experiments, however, must be done with caution due to putative differences in experimental conditions, such as transfection efficiencies of GFRα co-receptors, which may have an impact on the intensity readouts.

Table 5.1. Comparison of molar concentrations used in *in vitro* experiments in Studies III and IV.

Assay	Ligand	Concentration	Effect
RET phosphorylation in MG87RET fibroblasts	BT13	25-100 µM	+
	BT44	18-75 µM	+
	GDNF	6.6 nM	+
	NRTN	4.2 nM	+
Akt phosphorylation in MG87RET fibroblasts	BT13	25-100 µM	?
	BT44	36-75 µM	+
	GDNF	6.6 nM	+
	NRTN	4.2 nM	+
ERK phosphorylation in MG87RET fibroblasts	BT13	25-50 µM	+
	BT44	7.5-75 µM	+
	GDNF	6.6 nM	+
	NRTN	4.2 nM	+
Survival of cultured dopamine neurons (wt)	BT13	0.1-1 µM	+
	BT44	7.5 nM - 3.5 µM	+
	GDNF	0.33 nM	+
Survival of cultured dopamine neurons (RET knockout)	BT13	1-5 µM	–
	BT44	7.5-75 nM	–
	GDNF	0.33 nM	–
Protection of cultured dopamine neurons against MPP+ induced cytotoxicity (wt)	BT13	1 µM	+ (Mahato et al. 2020)
	BT44	75 nM	+
	GDNF	0.33 nM	+
Protection of cultured dopamine neurons against MPP+ induced cytotoxicity (RET knockout)	BT13	0.1-1 µM	– (Mahato et al. 2020)
	GDNF	0.33 nM	– (Mahato et al. 2020)

+, significant increasing effect compared to vehicle; –, no significant effects compared to vehicle; ?, no quantitative data but seems to increase phosphorylation compared to vehicle; wt, wild-type neurons

5.2.1 RET agonists phosphorylate RET and induce intracellular signaling cascades (III and IV)

A novel small molecule RET agonist BT13 stimulated the phosphorylation of RET in immortalized MG87RET fibroblasts transfected with GFRα1 or GFRα2 co-receptors as demonstrated by representative Western blot images (Figure 5.7.A and B). The effect of BT13 was concentration dependent and did not require the presence of GFRα co-receptors since RET phosphorylation was also elicited in GFP-transfected MG87RET fibroblasts incubated with BT13 (Figure 5.7.C). This suggests that BT13 functions as a direct RET agonist.

When the downstream effects of BT13 were investigated, we saw that RET phosphorylation activated the intracellular targets Akt and ERK which are imperative for neuronal survival and neurite outgrowth (Figure 5.7.D-F). Akt and ERK phosphorylation also seemed to be dependent on the concentration of BT13 but independent on the expression of GFRα1/2.

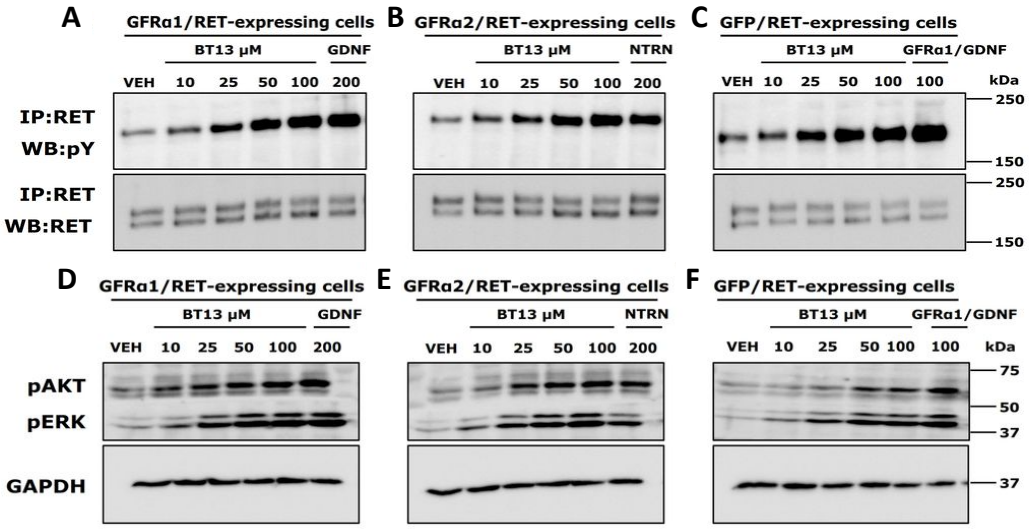

Figure 5.7. BT13 induces phosphorylation of RET and activates its downstream targets Akt and ERK in MG87RET fibroblasts. Representative Western blots show the increased level of RET phosphorylation in GFRα1 (**A**), GFRα2 (**B**) and GFP-transfected (**C**) cells in response to BT13 in a concentration-dependent manner. BT13 also increased the phosphorylation of Akt and ERK in a concentration-dependent manner in cells transfected with GFRα1 (**D**), GFRα2 (**E**) and GFP (**F**). GDNF, NTRN and soluble GFRα1/GDNF complex produced expected responses when used as positive controls for GFRα1, GFRα2 and GFP transfected cells, respectively. Their concentrations are provided in ng/ml. IP, immunoprecipitation; WB, Western blotting; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

In line with the *in vitro* data, BT13 (750 μg) increased the level of phosphorylated ERK (pERK) and ribosomal protein S6 (pS6; downstream target of Akt) when injected into the mouse striatum. pERK and pS6 immunolabelling was significantly enhanced in the BT13-injected striata as compared to the vehicle-injected striata (Figure 5.8.). GDNF (5 or 10 μg) was used as a positive control and produced a significant increase in pERK and pS6 staining in the striatum.

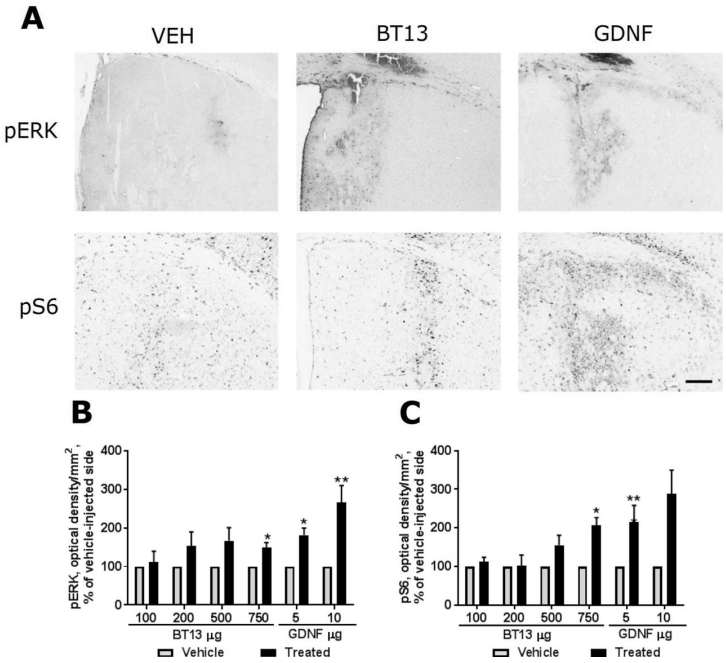

Figure 5.8. BT13 activates intracellular survival-promoting signaling pathways in the mouse brain. (**A**) Representative images of pERK and pS6 immunostained striatal sections collected after an intrastriatal injection of GDNF (10 µg), BT13 (750 µg) or vehicle. (**B**) Relative optical density of pERK labeling in the dorsal striatum as compared to the vehicle-injected side. BT13 (750 µg) increased the level of pERK (*p = 0.048; t(2) = 4.38). GDNF elevated the level of pERK at the dose of 5 µg (*p = 0.027; t(2) = 6.00) and 10 µg (**p = 0.009; t(3) = 6.19). (**C**) Relative optical density of pS6 labeling in the dorsal striatum as compared to the vehicle-injected side. BT13 (750 µg) increased the level of pS6 (*p = 0.040; t(3) = 3.47). GDNF elevated the level of pS6 at the dose of 5 µg (**p = 0.004; t(2) = 15.23). Significant differences between the vehicle and BT13/GDNF-injected sides were determined with paired two-tailed Student's *t*-test. Scale bar 200 µm; n = 3-4.

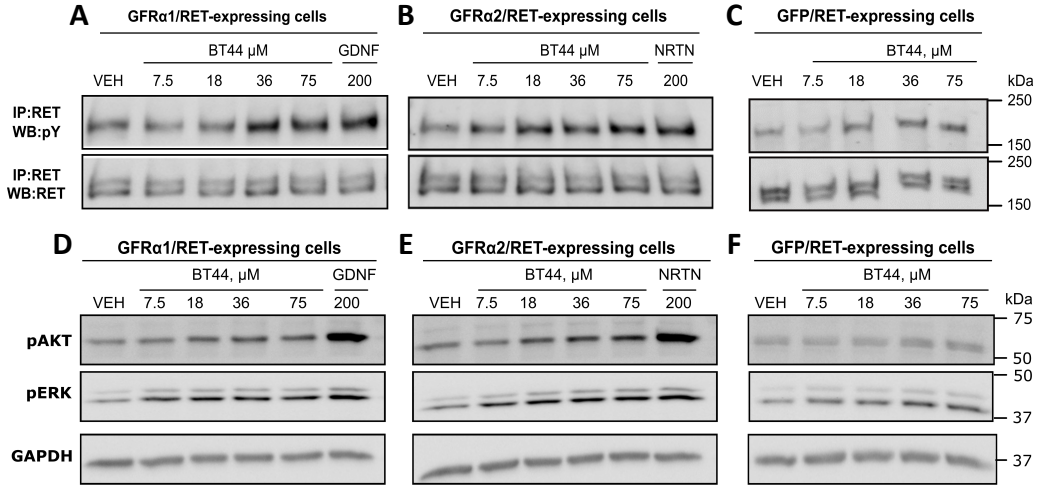

Figure 5.9. BT44 induces phosphorylation of RET and activates its downstream targets Akt and ERK in MG87RET fibroblasts. Representative Western blots show the increased level of RET phosphorylation in GFRα1 (**A**), GFRα2 (**B**) and GFP-transfected (**C**) cells in response to BT44 in a concentration-dependent manner. BT44 also increased the phosphorylation of Akt and ERK in a concentration-dependent manner in cells transfected with GFRα1 (**D**) and GFRα2 (**E**). In GFP-transfected cells, BT44 seemed to phosphorylate only ERK but not Akt (**F**). GDNF and NTRN (ng/ml) served as positive controls in cells transfected with GFRα1 and GFRα2, respectively. IP, immunoprecipitation; WB, Western blotting; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Analogously to the parental compound BT13, BT44 activated RET and its downstream target pathways in MG87RET fibroblasts. BT44 stimulated RET phosphorylation in cells transfected with the co-receptors GFRα1 and GFRα2 as well as in GFP-transfected cells suggesting a direct, co-receptor independent, agonistic mechanism (Figure 5.9.A-C). BT44 also induced the phosphorylation of Akt and ERK in GFRα1-RET and GFRα2-RET expressing fibroblasts (Figure 5.9.D and E). Interestingly, in GFP-transfected cells, BT44 induced ERK phosphorylation but did not have a clear effect on Akt phosphorylation (Figure 5.9.F).

5.2.2 RET agonists promote the survival of cultured midbrain dopamine neurons (III and IV)

Both BT13 and BT44 supported the survival of primary dopamine neurons *in vitro* as compared to vehicle. The number of TH-ir cells was increased in BT13 (0.1 and 1 µM), BT44 (7.5, 75 and 3500 nM) and GDNF (10 ng/ml ≈ 0.33 nM) -treated midbrain cultures from wild-type, but not from RET knockout, mice indicating that the survival-promoting effect of BT13 and BT44 on dopamine neurons was RET dependent (Figure 5.10.A-D). BT13 (1 µM) (Mahato et al. 2020) and BT44 (75 nM) also protected MPP+ -challenged dopamine neurons in culture with a similar efficacy as GDNF (10 ng/ml ≈ 0.33 nM) (Figure 5.10.E).

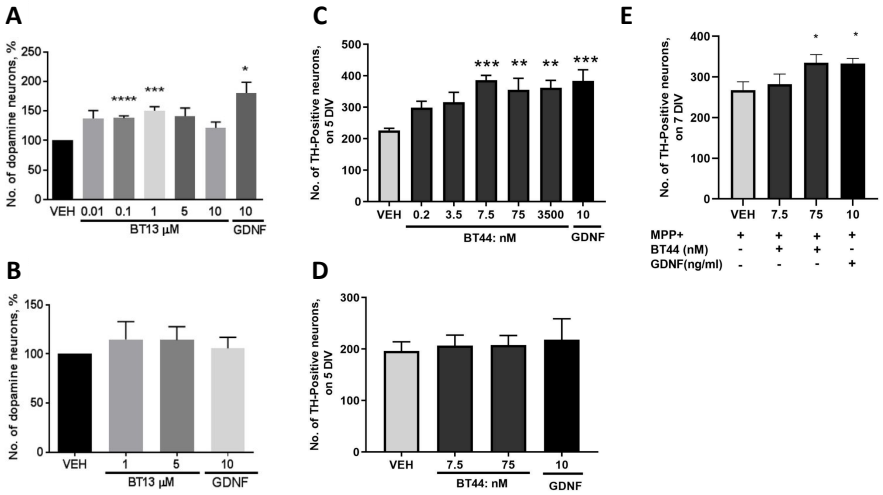

Figure 5.10. BT13 and BT44 promote the survival of cultured dopamine neurons from wild-type, but not from RET knockout, mice and protect them against MPP+ -induced cell death. (**A**) The number of TH-ir cells in wild-type midbrain cultures on 5th day *in vitro* (DIV) presented as percentage of the vehicle-treated samples. BT13 0.1 µM (****p < 0.0001), BT13 1 µM (***p = 0.0002) and GDNF 10 ng/ml (*p = 0.021; Dunnett after repeated measures ANOVA; $F_{2.35,16.46} = 6.55$, p = 0.006) increased the number of TH-ir neurons in the culture. N = 8. (**B**) The number of TH-ir cells in RET knockout midbrain cultures on 5th DIV presented as percentage of the vehicle-treated samples. N = 8. (**C**) The number of TH-ir cells in wild-type midbrain cultures on 5th DIV. BT44 7.5 nM (***p = 0.0008), BT44 75 nM (**p = 0.006), BT44 3500 nM (**p = 0.004) and GDNF 10 ng/ml (***p = 0.0009; Dunnett after repeated measures ANOVA; $F_{6,18} = 5.734$, p = 0.0018) increased the number of TH-ir neurons as compared to the vehicle. N = 4. (**D**) The number of TH-ir cells in RET knockout midbrain cultures on 5th DIV. N = 2. (**E**) BT44 75 nM (*p = 0.016) and GDNF 10 ng/ml (*p = 0.019; Dunnett after repeated measures ANOVA; $F_{3,15} = 5.410$, p = 0.01) increased the number of surviving TH-ir cells in wild-type midbrain cultures exposed to MPP+ neurotoxin. N = 6. Mean ± SEM; the number of TH-ir cells is normalized to the total number of cells in the culture.

5.3 Delivery of RET agonists into the striatum of hemiparkinsonian rats

5.3.1 BT13 protects against motor dysfunction in 6-OHDA model of Parkinson's disease (III)

The neuroprotective effect of intrastriatally infused BT13 was compared with that of GDNF in a unilateral 6-OHDA model of PD in rats. The experimental design is presented in Figure 4.2.A.

5.3.1.1 Motor deficits

Amphetamine-induced rotational behavior was similar in all treatment groups at two weeks following the toxin administration (Figure 5.11.A). In animals infused with BT13 (3-6 µg/24h) and GDNF (3 µg/24h), the number of ipsilateral turns was significantly reduced at four and six weeks after the lesion as compared to the vehicle infusion (Figure 5.11.B and C). Noteworthily, there was spontaneous recovery in the turning behavior of vehicle-treated rats at four ($p = 0.085$ for 2 weeks vs. 4 weeks: $t(11) = 1.895$) and six weeks post lesion ($p = 0.034$ and $p = 0.020$ for 2 weeks vs. 6 weeks: $t(10) = 2.460$ and 4 weeks vs. 6 weeks: $t(10) = 2.756$, respectively; paired two-tailed Student's *t*-test), an observation that is accordance with previously published data on this particular toxin administration scheme (Penttinen et al. 2016).

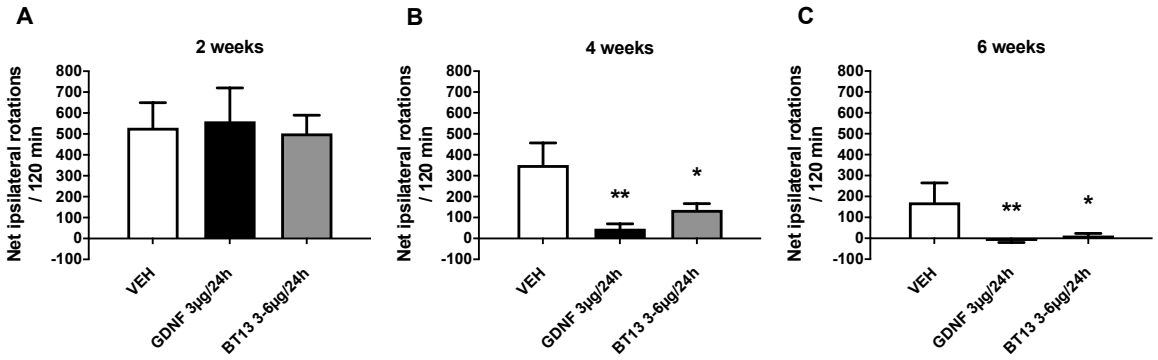

Figure 5.11. BT13 normalizes amphetamine-induced turning behavior in a time-dependent manner in 6-OHDA lesioned rats. (**A**) Effect of BT13 and GDNF infusions on amphetamine-induced turning behavior at two weeks post lesion. (**B**) At four weeks post lesion, the number of turns was lower in BT13 3-6µg/24h (*p = 0.013) and GDNF 3µg/24h (**p = 0.001) groups as compared to the vehicle group (Tukey HSD after one-way ANOVA; $F_{2,53} = 7.344$, $p = 0.002$). (**C**) The turning behavior was further reduced in BT13 3-6µg/24h (*p = 0.011) and GDNF 3µg/24h (**p = 0.007) groups as compared to the vehicle group at six weeks post lesion (Tukey HSD after one-way ANOVA; $F_{2,51} = 5.882$; $p = 0.005$). Mean ± SEM; vehicle n = 11-12, GDNF 3µg/24h n = 15-16, BT13 3-6µg/24h n = 25-27.

5.3.1.2 Dopaminergic neurons in the SNpc and fibers in the striatum

The immunohistochemical assessment of nigral and striatal sections collected after the last behavioral test revealed that GDNF 3 µg/24h infusion partially protected TH-ir fibers in the lesioned striatum as compared to the vehicle treatment (Figure 5.12.A and D). BT13 3-6 µg/24h, however, failed to produce a significant protective effect on dopaminergic fibers. Neither BT13 nor GDNF were able to protect TH-ir cells in the SNpc (Figure 5.12.B and E). Since GDNF delivery

into the striatum can downregulate the expression of TH (Georgievska et al. 2004; Salvatore et al. 2004), we used DAT as another dopaminergic marker to analyze the fiber density in the striatum. The density of DAT-ir fibers was comparable to that of TH-ir fibers in all treatment groups suggesting unaltered TH expression in the striatum (Figure 5.12.C and F).

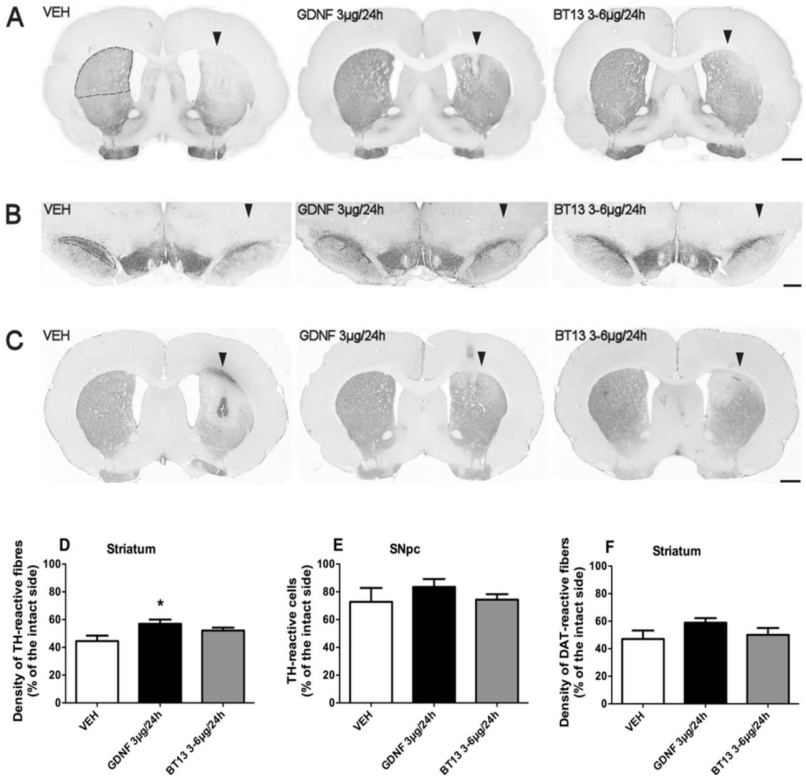

Figure 5.12. Effect of BT13 and GDNF on dopaminergic fibers in the striatum and cell bodies in the SNpc of 6-OHDA lesioned rats. Representative images of TH-ir fiber density in the striatum (**A**), TH-ir neurons in the SNpc (**B**) and DAT-ir fiber density in the striatum (**C**) in different treatment groups at six weeks post lesion. The dashed line in (A) shows the area in the dorsal striatum used for optical density measurements and in (B) the area used for stereological cell counting. The arrowheads denote the lesion side. (**D**) Quantification of TH-ir fiber density in the striatum. GDNF protected TH-ir fibers in the lesioned striatum as compared to the vehicle treatment (*p = 0.024; Tukey HSD after one-way ANOVA $F_{2,51} = 3.708$, p = 0.032). (**E**) Number of TH-ir cell bodies in the SNpc. (**F**) Densitometric quantification of DAT-ir fibers in the striatum. Scale bars: 1 mm (A and C) and 0.5 mm (B). Mean ± SEM; in TH staining (D and E) vehicle n = 11-12, GDNF 3µg/24h n = 15-16, BT13 3-6µg/24h n = 24-25; in DAT staining (F) vehicle n = 8, GDNF 3µg/24h n = 13, BT13 3-6µg/24h n = 19.

5.3.2 BT44 shows neurorestorative potential in 6-OHDA model of Parkinson's disease (IV)

The neurorestorative effect of intrastriatally infused BT44 was studied in a rat model of PD with progressive unilateral 6-OHDA lesion (Penttinen et al. 2016) utilizing amphetamine-induced rotational behavior and limb-use asymmetry tests. The experimental design is presented in Figure 4.2.B. Rats were assigned into equal treatment groups according to their amphetamine-induced rotation rate at 12 days after the 6-OHDA injections (Figure 5.13.A).

5.3.2.1 Motor deficits

Both GDNF 3 µg/24h and BT44 0.3 µg/24h were able to produce a functional recovery in 6-OHDA lesioned animals. Amphetamine-induced rotational asymmetry was significantly reduced in GDNF-infused rats at six and 12 weeks post lesion and in BT44 0.3 µg/24h -infused rats at 12 weeks post lesion as compared to the vehicle-treated rats (Figure 5.13.B and C). Spontaneous limb-use asymmetry in the cylinder test, however, was not balanced by any of the treatment infusions (Figure 5.13.E and F). This is in line with earlier literature where GDNF therapy has been reported to induce functional recovery in amphetamine-induced rotation test but not in the cylinder test (Georgievska et al. 2002; Gasmi et al. 2007b; Yue et al. 2014).

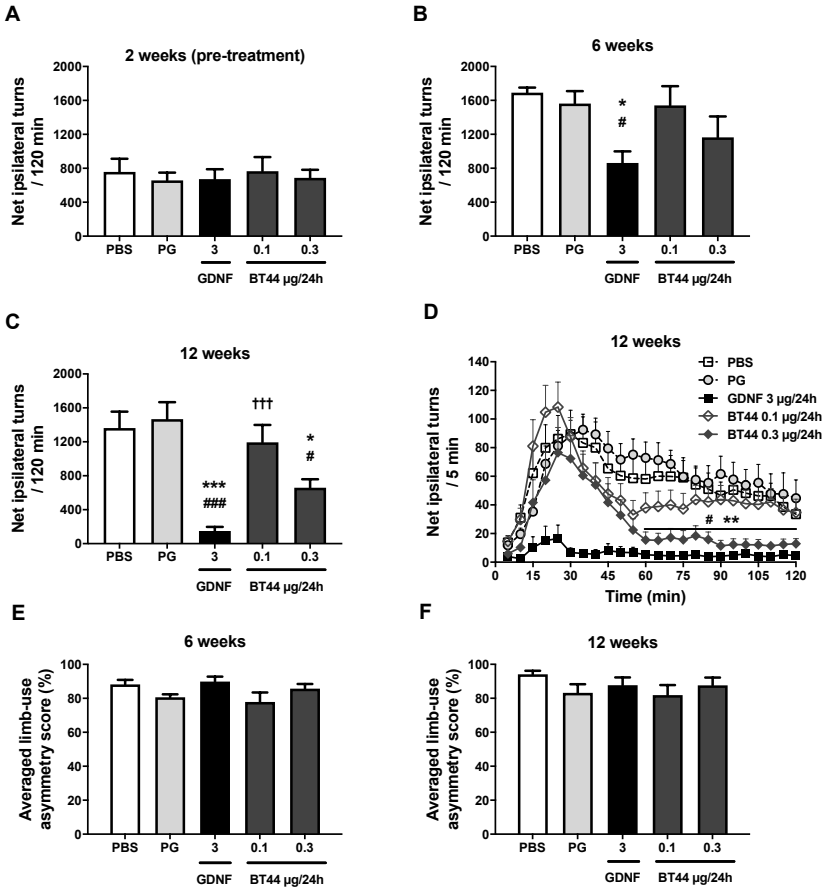

Figure 5.13. BT44 reduces amphetamine-induced rotations in 6-OHDA lesioned rats in a time-dependent manner. (**A**) Amphetamine-induced rotation rate at two weeks (12 days) post lesion was used to assigned rats into equal treatment groups. (**B**) At six weeks post lesion, GDNF 3µg/24h alleviated amphetamine-induced rotational asymmetry as compared to PBS (#p = 0.025) and PG (*p = 0.046; Tukey HSD after one-way ANOVA; $F_{4,45}$= 3.638, p = 0.012). (**C**) At 12 weeks, BT44 0.3µg/24h significantly reduced the number of ipsilateral turns as compared to PBS (#p = 0.046) and PG (*p = 0.010). GDNF-treated rats rotated significantly less than PBS (###p < 0.001), PG (***p < 0.001) and BT44 0.1µg/24h -treated rats (†††p < 0.001; Tukey HSD after one-way ANOVA; $F_{4,44}$= 11.365; p < 0.001). (**D**) Rotation rate per 5 min at 12 weeks post lesion. From 60-min time point onwards, the turning response was smaller in BT44 0.3µg/24h -infused rats as compared to PBS (#p = 0.029) and PG -infused rats (**p = 0.003; Tukey HSD after repeated measures ANOVA; 60–120 min: $F_{4,44}$= 8.491, p < 0.001). (**E-F**) There were no differences between the treatment groups in spontaneous limb-use asymmetry score measured in the cylinder test at six and 12 weeks post lesion. Mean ± SEM; n = 8-11.

Further analysis of the amphetamine-induced rotation rate per 5 min at 12 weeks post lesion revealed a distinct profile in the turning behavior of BT44 0.3 µg/24h -treated rats as compared to the other treatment groups (Figure 5.13.D). They seemed to have a faster and nearly full recovery from the strong amphetamine-response in the beginning of the experiment: from 60-min time point onwards, the rotation rate was significantly reduced in BT44 0.3 µg/24h group as compared to the vehicle groups.

5.3.2.2 Dopaminergic neurons in the SNpc and fibers in the striatum

The density of TH-ir fibers in the striatum was significantly higher in BT44 0.3 µg/24h and GDNF 3 µg/24h -infused rats than in PBS-infused animals (Figure 5.14.A). In accordance with this, BT44 0.3 µg/24h and GDNF 3 µg/24h seemed to increase the density of DAT-ir fibers in the striatum, but only the effect of GDNF 3 µg/24h was significant when compared to the vehicles (Figure 5.14.C). Intrastriatal infusion of GDNF, but not BT44, was also able to prevent the loss of dopamine neurons in the SNpc. The number of TH-ir and DAT-ir cell bodies in GDNF-treated rats was significantly higher as compared to the vehicle-treated rats (Figure 5.14.B and D).

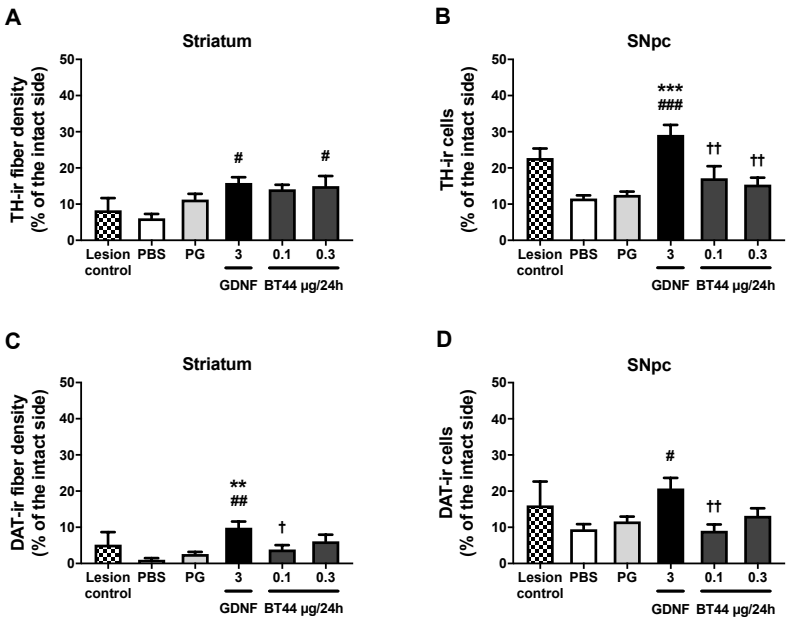

Figure 5.14. BT44 restores dopamine fibers in the striatum but is not able to protect dopamine neurons in the SNpc of 6-OHDA lesioned rats. (**A**) BT44 0.3µg/24h (#p = 0.025) and GDNF 3µg/24h (#p = 0.010) significantly increased the density of TH-ir fibers in the striatum as compared to PBS, while BT44 0.1µg/24h showed a trend for fiber restoration (p = 0.065; Tukey HSD after one-way ANOVA; $F_{5,46}$ = 3.671, p = 0.007). (**B**) The number of TH-ir cells was higher in the SNpc of GDNF-treated rats as compared to all other treatment groups (***p < 0.001 vs. PG; ###p < 0.001 vs. PBS; ††p < 0.01 vs. BT44 0.1µg/24h and BT44 0.3µg/24h; Tukey HSD after one-way ANOVA; $F_{5,48}$ = 8.898, p < 0.001). (**C**) The density of striatal DAT-ir fibers was higher in GDNF-treated rats as compared to PBS (##p = 0.001), PG (**p = 0.006) and BT44 0.1µg/24h -treated rats (†p = 0.046; Tukey HSD after one-way ANOVA; $F_{5,45}$ = 4.746, p = 0.001). (**D**) GDNF-treated rats had a greater number of DAT-ir cells in the SNpc than PBS (#p = 0.012) or BT44 0.1µg/24h -treated rats (††p = 0.008; Tukey HSD after one-way ANOVA; $F_{5,46}$ = 3.752, p = 0.006). The brains of the lesion control group were analyzed at two weeks post lesion to verify the extent of the lesion at the time of the treatment initiation. Mean ± SEM; lesion control group n = 4, other groups n = 8-11.

5.3.2.3 Correlation of TH immunohistological measures with amphetamine-induced rotational behavior

Due to the apparent discrepancies between amphetamine-induced turning behavior and TH immunohistological outcomes at 12 weeks post lesion, we analyzed the correlations of these measures quantitatively. Amphetamine-induced rotations showed low negative correlation with TH-ir fiber density in the striatum (Pearson's $r = -0.43$, $p < 0.01$) and moderate negative correlation with TH-ir cell count in the SNpc (Pearson's $r = -0.64$, $p < 0.001$) when data from all treatment groups were pooled together (Figure 5.15.A and B). Noteworthily, GDNF-treated rats rotated substantially less as compared to all other rats, but this behavioral improvement was not accompanied by fiber restoration in the striatum to the same extent. This is visualized in Figure 5.15.A as a grouping of GDNF data points (black squares) close to the y-axis but not higher than the other data points. We also analyzed the correlation between the striatal fiber densities and nigral cell numbers from the pooled dataset and found a low correlation between these two histological read-outs (Pearson's $r = 0.45$, $p < 0.01$) (Figure 5.15.C).

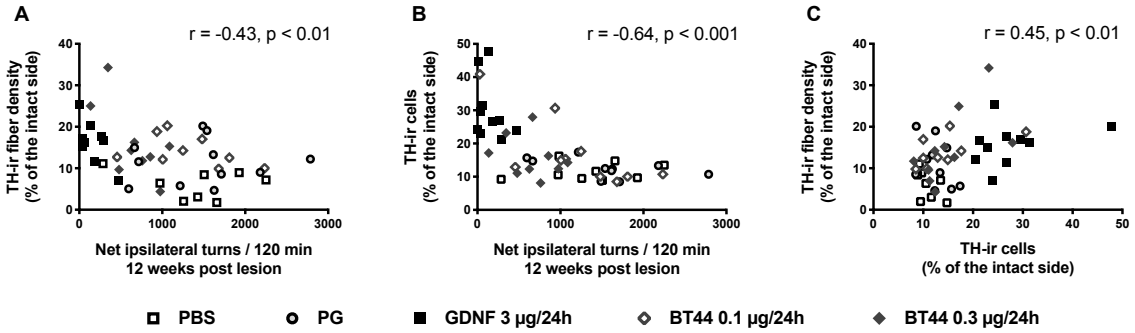

Figure 5.15. Correlations between TH immunohistochemical measures and amphetamine-induced turning rate. (**A**) Amphetamine-induced turning rate at 12 weeks post lesion plotted against striatal TH-ir fiber density for each experimental animal. (**B**) Amphetamine-induced turning rate at 12 weeks post lesion plotted against nigral TH-ir neuronal number for each experimental animal. (**C**) TH-ir cell numbers in the SNpc plotted against TH-ir fiber densities in the striatum. r - Pearson correlation coefficient, all treatment groups analyzed together.

6 DISCUSSION

This work aimed to support the preclinical characterization of neurotrophic therapies for PD by testing the effects of unconventional NTFs, CDNF and MANF, in the non-lesioned rat brain and novel small molecule RET agonists, BT13 and BT44, in an animal model of PD. Patients await a disease-modifying treatment for their debilitating condition. Therefore, efforts have to be made to overcome the lack of translatability of NTF-based treatments that has overshadowed the field of research until today.

The main outcomes suggest that exogenously administered GDNF, CDNF and MANF divergently modify dopamine release and dopamine synthetizing and metabolizing enzymes in the nigrostriatal system of the normal rat brain. We saw that nigrally delivered CDNF readily diffuses around the brainstem and colocalizes with TH-ir, but not PV-ir, neurons in the SN. No active transport of CDNF into distal brain areas was detected after a nigral injection. BT13 and BT44 were shown to activate RET and its downstream intracellular target pathways responsible for neuronal survival and regeneration *in vitro*. They also supported the survival of cultured midbrain dopamine neurons in a RET-dependent manner. BT13 promoted functional recovery in a neuroprotection model of PD, and BT44 alleviated motor impairment and showed potential to restore striatal dopaminergic fibers in a neurorestoration model of PD in rats.

6.1 Effects of exogenously administered neurotrophic factors on dopamine release in the striatum

A single intrastriatal injection of 10 µg of GDNF, CDNF and MANF has been shown to produce robust neuroprotective effects on nigrostriatal dopamine neurons at four weeks after the injection (Lindhom et al. 2007; Voutilainen et al. 2009). Also in normal rats, a single 10 µg -injection of GDNF into the SNpc was shown to elicit neurochemical changes on dopaminergic system that persisted for at least three weeks (Hudson et al. 1995). We set out to characterize the effects of GDNF, CDNF and MANF on dopaminergic neurotransmission at one and three weeks after the striatal delivery. Importantly, our experiments were conducted in freely-moving animals in order to avoid the confounding effects of anesthetics on neuronal functions (Marinelli and McCutcheon 2014; Müller et al. 2011). Our brain microdialysis results provide the first insight into the long-lasting and divergent biological effects of exogenously administered CDNF and MANF on nigrostriatal dopamine system in the normal rat brain. These are relevant data when considering these NTFs as a potential therapeutic approach for PD. It is important to confirm that they do not decrease the release of dopamine in the striatum, and therefore have apparent interactions with the dopaminergic medications of PD.

We discovered that an intrastriatal injection of MANF significantly elevated stimulus-evoked dopamine output and enhanced DOPAC/dopamine turnover in the striatum one week after the injection. MANF may modulate presynaptic release mechanisms or storage pools of dopamine resulting in enhanced neurotransmission. Using two distinct stimuli (high concentration of K^+ and

amphetamine) to evoke dopamine release, we pursued to dissect the contribution of these factors to the final output. Hypertonic K^+ solution depolarizes nerve terminals and causes Ca^{2+}-dependent exocytosis of vesicles close to the presynaptic membrane (Westerink et al. 1989). This pool of presynaptic dopamine is considered to be readily releasable. Amphetamine stimulus, on the contrary, gives an estimate of the total amount of dopamine stored in the synaptic terminals by depleting vesicular dopamine stores and causing Ca^{2+}-independent release of dopamine (Westerink et al. 1989; Sulzer 2011). We saw an increase both in potassium- and amphetamine-evoked dopamine overflow in MANF-injected rats. Thus, MANF seems to replenish presynaptic dopamine stores and enhance Ca^{2+}-mediated exocytosis or increase the proportion of readily releasable pool of vesicles. In primary neuron cultures and acute brain slices, GDNF has been shown to facilitate synaptic transmission by increasing the quantal size of dopamine release, potentiating Ca^{2+} influx and inhibiting A-type K^+ channels (Pothos et al. 1998; Bourque and Trudeau 2000; Yang et al. 2001; Wang et al. 2003). It is possible that MANF exerts corresponding modulatory effects on the synaptic terminals. The spatiotemporal resolution of microdialysis, however, is not good enough to draw conclusions about precise mechanisms behind the observed effects. For that, further studies focusing on the cellular-level mechanisms are needed. Studies with *in vivo* voltammetry would also offer a better temporal and spatial resolution to clarify the mechanisms of enhanced dopamine release in MANF-treated rats.

As discussed below in chapter 6.4. MANF and CDNF have better diffusion properties in the brain parenchyma as compared to GDNF (Mätlik et al. 2017; Voutilainen et al. 2011, 2009). They can reach dopaminergic terminals in the whole striatum and enhance their function which can contribute to the overall dopamine outflow. GDNF, instead, readily binds to the heparan sulfate side chains of the extracellular matrix which mostly likely restricts its distribution close to the injection site and reduces the effects on distant nerve terminals. Increased sprouting of dopaminergic fibers in the striatum could also account for the enhanced dopaminergic neurotransmission in MANF-treated animals, but this seems unlikely since an earlier study demonstrated no effect of intrastriatal infusion of MANF on TH-ir fibers in the striatum of intact rats (Voutilainen et al. 2011).

Microdialysis is a highly invasive method causing mechanical damage and gliosis around the sampling site (Benveniste and Hansen 1991). ER stress and inflammation are inevitable consequences of the mechanical insult. The capability of MANF and CDNF to mitigate ER stress and neuroinflammation could partly explain their augmented effects as compared to GDNF in the microdialysis experiment. Furthermore, the implantation of the guide cannula after the NTF injection disrupts the BBB. Hypothetically, this enables NTF-neutralizing antibodies to invade to the brain which can cause unexpected variation to the effects of the NTFs.

It was unexpected that GDNF did not enhance stimulus-evoked dopamine release. By contrast, CDNF had a more pronounced effect than GDNF in spite of the fact that the half-life of exogenous GDNF in the brain has been estimated to be 3-4 days whereas the half-life of CDNF is only approximately 5.5 h (Granholm et al. 2000; Mätlik et al. 2017). We showed that COMT activity was increased after GDNF injection. It can be speculated that the smaller effect of GDNF on stimulus-evoked dopamine release results from the enhanced dopamine metabolism which contributes to

the clearance of extracellular dopamine. GDNF/RET signaling is also shown to function as a negative regulator of the cell surface trafficking of DAT (Boger et al. 2007; Kopra et al. 2017; Littrell et al. 2012; Zhu et al. 2015). This could partly explain why amphetamine-stimulated dopamine release was not enhanced in GDNF-treated rats.

The recombinant NTFs used in Study I were produced in different cell lines which might have contributed to their divergent effects. GDNF was produced in *E. coli*, CDNF in Sf9 insect cells and MANF in mammalian Chinese hamster ovary cells. NTFs produced in mammalian cells may have stronger activity than NTFs produced in other cell lines. Proteins produced in insect or mammalian cells can be post-translationally glycosylated unlike proteins produced in bacterial cell lines. Glycosylation has an impact on the biological properties of recombinant proteins. According to mass spectrometer analysis, however, CDNF and MANF used in our experiment were not glycosylated making them comparable with GDNF in that regards.

Finally, it is tempting to speculate that the differences seen in dopamine release may originate from other neurotransmitter systems. NTFs may well affect, for example, cholinergic interneurons or glutaminergic terminals in the striatum. Increased activation of presynaptic nAChRs or NMDA receptors on dopaminergic nerve terminals would facilitate the release of dopamine. Thus, it would be informative to measure stimulus-evoked release of other neurotransmitters such as glutamate and acetylcholine in the striatum after the injection of NTFs.

6.2 GABA release in the globus pallidus after exogenously administered neurotrophic factors

An essential question that remains is what are the effects of NTFs on other neuronal populations outside the nigrostriatal dopamine pathway (Aron and Klein 2011). The non-dopaminergic effects are equally important to be explored because they might cause adverse events in PD patients or give rise to positive therapeutic benefits alleviating, for example, the non-motor symptoms of PD. Despite its importance, this area of research has been overshadowed by the studies focusing on the dopaminergic effects of NTFs. The effects of NTFs on the GABAergic system, for instance, are substantive since they directly affect the output of the basal ganglia and motor control. Salvatore and colleagues (2009) reported long-lasting effects of a single unilateral injection of GDNF on striatal proteins regulating GABAergic neuronal function in the non-lesioned rat brain. They described for example bilateral changes in GABA synthesizing enzyme glutamic acid decarboxylase (GAD) 65/67 and increased levels of D1 receptor and phosphorylated DARPP-32 (dopamine and cAMP regulated phosphoprotein 32 kDa) in the contralateral striatum after the striatal delivery of GDNF. Patch clamp recordings from rat midbrain slices suggest that MANF can acutely increase GABA release and $GABA_A$ receptor-mediated inhibitory postsynaptic currents in dopamine neurons of the SNpc (Zhou et al. 2006). The physiological relevance of these phenomena, however, has remained obscure.

To address the question whether GDNF, CDNF and MANF have divergent effects also on GABAergic MSNs, we performed another microdialysis experiment where we measured potassium-evoked release of GABA in the striatopallidal terminal area GPe and striatonigral terminal area SNr one

and three weeks after an intrastriatal NTF injection. K^+-stimulus caused a marked increase in the extracellular concentration of GABA in the GPe, but we did not detect significant differences between the treatment groups at either of the time points. Unfortunately, GABA concentration in the dialysates collected from the SNr was too low to be quantified reliably with our HPLC system. In the future experiments probing the SNr, either the perfusion rate of the dialysis solution has to be slower or the dialysis membrane has to be longer to yield higher concentration of GABA in the samples. No-net-flux microdialysis would also permit the determination of the actual extracellular concentration of GABA at the baseline. However, this method is not suitable for the detection of stimulus-evoked rapid concentration changes.

Due to the lack of significant differences between the treatment groups, speculations on the possible effects of NTFs on GABAergic neurotransmission are avoided here. Overall, this pilot study should be repeated in order to draw reliable conclusions from the results.

6.3 Effects of exogenously administered neurotrophic factors on dopamine synthesis and metabolism

We provided novel data on the effects of CDNF and MANF on TH activity and the effect of GDNF on the activity of dopamine metabolizing enzymes COMT and MAO. To clarify if the elevated dopamine release in MANF-injected rats was due to enhanced synthesis of dopamine, we measured *in vivo* TH activity in the striatum one week after the intrastriatal NTF delivery. We saw significantly increased TH activity only in GDNF-injected rats. This observation is in accordance with earlier results showing increased TH phosphorylation and enzymatic activity in rats administered with exogenous GDNF or MEN2B mice with constitutively active RET (Beck et al. 1996; Rosenblad et al. 2003; Georgievska et al. 2004; Salvatore et al. 2004; Mijatovic et al. 2008; Salvatore et al. 2009). Perhaps, out of the NTFs studied here, only GDNF has the ability to stimulate the phosphorylation of TH. It should be noted, however, that we investigated only one time point, and therefore the data are not sufficient to draw conclusions whether the NTFs can modify TH activity at different time points. It would be worth testing if MANF and CDNF are also able to modify the phosphorylation of TH as well as the time-dependence of the effects of NTFs on TH activity. Taken together, TH activity data could not provide an explanation for the increased stimulus-evoked dopamine overflow seen in MANF-treated rats. Other possible mechanisms are discussed above in chapter 6.1.

An intriguing outcome from the microdialysis experiment was the significantly decreased DOPAC/HVA ratio in GDNF-injected rats. This phenomenon triggered us to further elucidate the effect of GDNF on the activity of COMT, MAO-A and MAO-B. Indeed, we detected increased COMT activity in the striatum of GDNF-injected rats together with reduced MAO-A activity which was, perhaps, a compensatory response to the increased COMT activity. This finding is well in line with an earlier study where a 10-µg nigral injection of GDNF elicited a dose-dependent increase in dopamine turnover measured as HVA/dopamine ratio in the SN and striatum tissue samples one week after the injection to normal rats (Hudson et al. 1995). The observed changes in COMT and MAO-A activity direct dopamine degradation towards 3-MT-mediated pathway and away from DOPAC-mediated pathway giving a logical explanation for why DOPAC was formed relatively less

as compared to HVA. The putative changes in 3-MT levels would help to further clarify the effects of NTFs on the metabolic pathways of dopamine, but our HPLC system was not optimized to detect 3-MT in the dialysates or tissue samples.

LPS-induced microglia activation has been shown to enhance total COMT activity in the rat brain (Helkamaa et al. 2007). In our study, we controlled the possibility for microglia activation due to the surgical procedure by comparing the vehicle-injected striatum to the non-injected striatum. No differences in COMT activity between the two hemispheres were detected. Thus, the observed effect on COMT activity is not due to the injection procedure *per se*, but rather related to the injected GDNF solution. However, the rhGDNF used in the study was produced in an *E. coli* cell line. Although it was tested by the manufacturer to be compliant for cell culture use in terms of LPS counts, we cannot exclude the possibility that there were some bacterial LPS residues in the product which could induce microglia activation and result in enhanced COMT activity. To confirm the results, GDNF originating from a mammalian cell line should be tested in the same setup and microglia activation after GDNF-injection should be ruled out using e.g. Iba1 (ionized calcium-binding adapter molecule 1) immunohistochemistry. Furthermore, follow-up experiments should test whether GDNF injection has an effect on the expression level of COMT which could contribute to the increased enzyme activity. The time-dependence of COMT activation and the effects of MANF and CDNF on the activity of dopamine metabolizing enzymes would also be interesting to investigate in the future.

The neurotrophic therapies should be compatible with the current PD medications that are described in chapter 2.3.5 and Table 2.2. Thus, the increased COMT activity after GDNF treatment is an important aspect to take into consideration if GDNF is administered to PD patients. This might call for adjustments to the existing dopamine-replacing drugs; for example, the dosage of a COMT inhibitor might need to be increased. The enhanced *in vivo* TH activity in GDNF-treated rats and elevated stimulus-evoked dopamine release in MANF-treated rats, on the other hand, could translate into reduced L-DOPA doses in PD patients.

6.4 Spreading properties of neurotrophic factors after administration into the basal ganglia

Insufficient brain distribution and bioavailability have limited the clinical benefits of the NTFs tested in phase II trials (Lapchak et al. 1998; Hamilton et al. 2001; Salvatore et al. 2006). Knowing the diffusion and transportation properties of a neurotrophic treatment is of paramount importance when delineating the optimal site of administration for a therapy that is intracranially delivered to a single site in a condition which afflicts normal neuroanatomical connections and is accompanied with disturbed axonal transport (Chu et al. 2012; De Vos et al. 2008; O'Keeffe and Sullivan 2018). Intrastriatally administered MANF has been shown to diffuse significantly better in the rat brain when compared to GDNF (Voutilainen et al. 2011, 2009). CDNF also diffuses readily and spreads to the cortical areas and hippocampus after an injection or infusion into the striatum (Mätlik et al. 2017; Voutilainen et al. 2011). GDNF and CDNF have been demonstrated to undergo retrograde transport to the SNpc along nigrostriatal dopamine neurons when injected into the striatum (Tomac et al. 1995b; Voutilainen et al. 2011; Mätlik et al. 2017). MANF, instead, is seems

to be actively transported from the striatum to the frontal cortex but not to the SN (Voutilainen et al. 2009).

Our pilot experiment suggested that a 3-µg injection of CDNF into the SN had comparable effects with a 10-µg striatal injection of CDNF on behavioral and histological read-outs in a 6-OHDA rat model of PD (unpublished data). Therefore, we wanted to further examine how CDNF behaves when injected into the SN; how well it diffuses into the surrounding brain areas, how different variables affect the diffusion properties, and is it transported or taken up by specific types of neurons. As expected, CDNF had a widespread diffusion in the brainstem of normal rats after the nigral injection. CDNF immunolabelling was detected at 2 and 6 h, but not anymore at 24 h post injection. This suggests that the exogenous CDNF was degraded between 6 and 24 h which goes well in line with the 5.5-h half-life of CDNF (Mätlik et al. 2017). The volume of distribution of injected CDNF was increased with the increased injection volume which is clearly an important factor to be optimized when the delivery protocol for a therapeutic protein is being established. A larger injection volume results in the diffusion of the protein to more distant brain regions which may give rise to enhanced therapeutic effects or emerge of adverse effects depending on the protein in question, the affected brain regions and the clinical condition.

We saw similar punctate CDNF-ir staining inside the TH-ir neurons of the SNpc as was previously shown after striatal injection and retrograde transportation of CDNF to the SNpc (Mätlik et al. 2017). CDNF did not seem colocalize with PV-ir neurons in the SNpc suggesting selective uptake into dopamine neurons. This can possibly contribute to the neuroprotective effects of CDNF on dopamine neurons and its favorable safety profile. Further experiments with quantitative outcomes, however, are needed to draw decisive conclusions of the localization of exogenously administrated CDNF.

When radiolabeled CDNF was injected into the SN we observed prominent spread to the ipsilateral STN, but no active transport to the striatum or other distal brain areas. It is possible that upon cellular uptake CDNF is targeted to lysosomal degradation instead of axonal transportation when injected near the cell bodies as is the case with NGF (Butowt and von Bartheld 2001). Noteworthily, only one time point was analyzed after the injection of radiolabeled CDNF. As seen in the diffusion experiment, there is little CDNF immunoreactivity left in the brain at 24 h post injection, probably due to the short half-life of CDNF. It is possible that CDNF is transported from the SN but not detected in other brain areas 24 h after the injection anymore. Thus, it would be good to check if there are signs of active transport at earlier time points (e.g. at 2 h and 6 h) after the injection. Co-administration of unlabeled CDNF did not significantly reduce the radioactive signal in the STN suggesting that CDNF was passively diffused to this proximal nucleus instead of actively transported. Active transportation, however, cannot be completely ruled out because the prominent diffusion of radiolabeled CDNF to the area covering the STN potentially masks the effect of the transportation.

6.5 Potential of neurotrophic factor mimetics as a therapeutic strategy for Parkinson's disease

Since the clinical proof-of-concept studies with NTFs have remained inconclusive, it is pivotal to look for novel neurotrophic strategies to protect the degenerating dopamine neurons and restore the impaired functionality of basal ganglia circuitry. There are several protein therapy related challenges that may limit the therapeutic use of NTFs for PD or other brain diseases. Firstly, proteins do not cross the BBB and therefore require surgical delivery into the brain which increases the costs and risks of the treatment. In the clinical studies with GDNF and AAV2-NTRN, the most common safety concerns have related to the intracranial drug delivery procedure and implantation of the infusion device (Lang et al. 2006; Marks et al. 2010; Whone et al. 2019). Secondly, the production of biological therapeutics for clinical use is not trivial. Special attention has to be paid to the batch-to-batch variations and stability of the recombinant protein. Additionally, the levels host cell contaminants or endotoxins (such as LPS) have to be carefully controlled. Thirdly, formation of neutralizing antibodies against the recombinant NTF treatment is a particular concern (Lang et al. 2006; Heiss et al. 2019). Anti-NTF antibodies can potentially limit the efficacy of the therapeutic protein and cross-react with the corresponding endogenous NTF causing its loss-of-function. Finally, low bioavailability and poor tissue penetration are well-known issues with the GFL-therapeutics as discussed in the previous chapter. Small molecules that retain the neurotrophic activity of NTFs by activating the same receptors and signaling pathways provide an attractive approach to circumvent these protein therapy related challenges.

Using virtual and cell-based screening methods a novel family of small molecule GFL mimetics, including BT13 and BT44, has been identified and optimized (Saarma et al. 2014; Sidorova et al. 2017). These compounds seem to diffuse readily in the brain parenchyma and penetrate the BBB. They activate GFL-GFRα-RET signaling complex, but the exact mechanism of action is not elucidated yet. Molecular docking simulations have identified two possible binding sites: the putative allosteric modulation site in GFRα or the GFRα-interfacing surface of RET (Ivanova et al. 2018). The latter would imply that the compounds act as a direct RET agonists. Recent results support their function as direct RET agonists because of their ability to phosphorylate RET and activate downstream signaling cascades also in the absence of GFRα co-receptors (Sidorova et al. 2017; Viisanen et al. 2020; Study III; Study IV). BT13 and BT44 are first-in-class molecules with drug-like properties that selectively (see below) activate RET and its downstream survival-promoting signaling pathways *in vitro*. This work demonstrated that they can markedly alleviate amphetamine-induced rotational behavior in a unilateral 6-OHDA lesion model of PD in rats. BT44 also showed potential for restoring TH-ir fibers in the striatum of hemiparksinonian rats, but the neuroprotective effects on dopaminergic cell bodies in the SN remained unattained. BT13 and BT44 serve as promising lead compounds; with further optimization they can be developed into a novel disease-modifying therapy for PD.

The potential off-target effects of BT44 were assessed in a panel of *in vitro* assays by an external company (Eurofins CEREP SA, France, see details Study IV supplementary data). BT44 (1 μM) did not affect the activity of selected ion channels, G-protein coupled receptors, transporters, kinases or dopamine metabolizing enzymes. In all assays, the target inhibition or stimulation was less than

25% which, according to the company's guidelines for result interpretation, reflects assay variability and indicates the lack of a significant effect of the test compound. Our data also show that BT44 only supports the survival of RET-expressing wild-type, but not RET knockout, dopamine neurons and fails to activate ERK-related signaling in TrkB-expressing murine fibroblasts lending support to the selectivity of BT44 for RET.

BT44 promoted functional recovery in 6-OHDA lesioned rats. When we analyzed the amphetamine-induced turning rate per 5 min at 12 weeks post lesion, we saw an interesting feature in BT44 0.3 µg/24h -treated animals. They showed divergent time-dependence of the rotational asymmetry and seemed to recover faster from the amphetamine challenge when compared to other treatment groups. It can be speculated that BT44 alters dopamine dynamics. For example, reduced dopamine uptake through DAT or activity of dopamine metabolizing enzymes could account for slower clearance of dopamine from the extracellular space of the lesioned striatum, and consequently faster recovery from the amphetamine-induced turning response. Important to note that in Study III, BT13 was shown to acutely increase the level of extracellular dopamine in the dorsal striatum in mice. As mentioned earlier, RET signaling is suggested to negatively regulate DAT function and/or cell surface trafficking (Boger et al. 2007; Kopra et al. 2017; Littrell et al. 2012; Zhu et al. 2015). Moreover, it is possible that as a lipophilic compound, BT44 is able to diffuse into the non-lesioned hemisphere during the long infusion period where it could augment dopamine clearance from the extracellular space, for example by enhancing COMT-mediated degradation of dopamine as was shown to happen after GDNF administration in Study I. Further experiments assessing, for example, the effects of BT44 on DAT or COMT activity or its diffusion into the contralateral striatum are needed to explain the altered rotation rate profile.

In the *in vivo* experiments, we encountered solubility problems related BT-compounds; they were insoluble to aqueous vehicles forcing us to use PG as a vehicle. Even with PG, the solution required heating and sonication in order to get the compounds thoroughly dissolved. We cannot be sure if any precipitation occurred in the infusion pumps or when the solution got in contact with aqueous extracellular fluid. These solubility issues should be resolved before proceeding to further *in vivo* experiments. The high-resolution 3D structure of GFRα1 and the model for GDNF-GFRα1 interaction provide an opportunity for rational structure-based drug design (Leppänen et al. 2004). Novel molecules capable of binding to specific GFRα receptors with improved drug-like properties could help to overcome some of the difficulties related to the current BT compounds. After lead optimization, either BT-scaffold-based compounds or new GFL mimetics could be suitable for systemic administration. Compared to intracranial delivery it would be a less risky approach for large-scale clinical use and would help to overcome the problematic question about the most effective delivery site in the brain. However, the peripheral expression of GFRα and RET receptors should be considered when systemic administration of GFL mimetics is planned. Adverse effects can occur as a result of RET activation for example in the PNS, testis or thyroid gland.

Apart from direct RET agonists, an attractive therapeutic approach for harnessing RET signaling pathways would be to develop selective positive allosteric modulators (PAMs) of GFRα1. The first compound possessing such biological activity, XIB4035, has been described, but not tested in

animal models of PD (Tokugawa et al. 2003; Hedstrom et al. 2014). GFRα1-PAMs would potentiate the trophic effects of endogenous GDNF and provide major advantages over direct RET agonists: Firstly, they could reduce the risk of RET-induced adverse effects as compared to exogenous GDNF or direct RET agonists. The activation of RET would only occur in the presence of endogenous GDNF preserving the homeostatic regulation mechanisms of GDNF-GFRα1-RET signaling, and administration of unphysiological concentrations of GDNF would not be needed. Secondly, they could provide superior selectivity as compared to direct RET agonists owing to the limited expression of particular GFRα–RET pairs in different brain areas and other organs (Trupp et al. 1997; Golden et al. 1998). Lastly, binding of a compound directly to RET on the surface interfacing with GFRα1 can potentially disrupt the signaling of endogenous GDNF. This partial antagonism could explain the submaximal efficacy of BT44 as compared to GDNF in our neurorestoration experiment (Study IV). In addition, continuous RET activation during the treatment infusion period might have caused downregulation of the receptor. In this case, subsequent withdrawal of the RET agonist would lead to an acute depletion of trophic support contributing to the lack of neuroprotective effect on nigral neurons.

6.6 Methodological considerations

6.6.1 Considerations relating to 6-OHDA lesion models

In our neuroprotection experiment (Study III) evaluating the efficacy of BT13 in hemiparkinsonian rats, a single unilateral injection of 6-OHDA (16 µg) into the dorsal striatum resulted in relatively mild dopaminergic degeneration: in vehicle-treated rats, the density of TH-ir fibers in the striatum was reduced approximately by 55% and the number of TH-ir neurons in the SNpc by 27%, which correspond to the estimated state of the nigrostriatal system in PD patients at the onset of the motor symptoms, i.e. at early stage of the disease (Burke and O'Malley 2013). The lesion size was similar than the "regressive" lesion described by Penttinen et al. (2016). Because the size of the lesion was at the threshold of the motor manifestations, small changes at cellular level may have caused disproportionally profound functional improvement as seen with the almost completely vanished rotational behavior in GDNF and BT13 -treated rats (Björklund and Dunnett 2019). We also saw spontaneous recovery in terms of rotational behavior in vehicle-treated animals. This phenomenon is well-documented in the literature for rats with a small (0-30% loss of the SNpc neurons) or medium (30-75% loss of the SNpc neurons) 6-OHDA lesion (Stanic et al. 2003; Stanic et al. 2003b; Penttinen et al. 2016). The clear amphetamine-induced turning bias observed after two weeks is recovered by 10-16 weeks post lesion in animals with a medium-size lesion and even faster in animals with a small lesion. This functional recovery is associated with axonal regrowth, sprouting and phenotypic recovery of the nigrostriatal dopamine neurons (Blanchard et al. 1996; Finkelstein et al. 2000; Stanic et al. 2003). Spontaneous recovery is a confounding factor in our neuroprotection experiment implicating that the functional effects of BT13 have to be interpreted with adequate caution. The lack of effect on TH-ir and DAT-ir fibers in the striatum reinforces the view that the reduction in amphetamine-induced rotational behavior is not due to neuroprotection. Perhaps, a larger 6-OHDA lesion could have produced a more stable lesion with a better correlation between the histological and functional read-outs.

In the next experiment (Study IV), we wanted to utilize neurorestoration paradigm with a much more severe 6-OHDA lesion. In the neurorestoration model, it is possible to balance animals into experimental groups according to baseline rotation scores after the lesion (Figure 4.2.). This helps to control interindividual variation and reduce the groups sizes needed to see therapeutic effects. Of note, as BT13 was speculated to regulate DAT activity (Study III), we wanted to make sure that BT44 could not directly interfere with the neurotoxic effect of 6-OHDA which is dependent on DAT-mediated uptake into dopamine neurons. Most importantly, the delivery of the experimental compounds at the time when the lesion is already established reflects much better the clinical context in PD making the neurorestoration paradigm a more relevant model as compared to the neuroprotection paradigm. In addition, we wanted to produce a bigger and more stable lesion in order to get rid of spontaneous recovery and provide a larger window for the experimental therapeutics to produce their effects.

The 6-OHDA delivery paradigm employed in our neurorestoration experiment, indeed, resulted in a much more severe lesion in agreement with the earlier results (Penttinen et al. 2016). In the lesion control group, the density of TH-ir fibers in the striatum was reduced approximately by 90% and the number of TH-ir cell bodies in the SNpc by 77% at two weeks post lesion. The number of TH-ir cells in the SNpc of PBS and PG -treated animals at 12 weeks post lesion was significantly lower as compared to the lesion control group indicating that the dopaminergic degeneration was not fully developed at the time of the treatment initiation but rather retrogradely progressing. It is well known that striatal denervation occurs rapidly after 6-OHDA injection into the striatum, but the cell bodies in the SNpc die gradually over several weeks or months (Björklund et al. 1997). The lesion size in the neurorestoration experiment reflects the state of the nigrostriatal system in advanced PD, i.e. the status of patients when intracerebral treatment procedures can be considered (Kordower et al. 2013). The neuronal damage was accompanied by persistent motor deficits as indicated by prominent amphetamine-induced rotational asymmetry and decrease in spontaneous contralateral forepaw use in the cylinder test in vehicle-treated rats. Regardless of the severe lesion, BT44 was able to induce delayed functional recovery in turning behavior and show small, but significant, restorative effect on dopaminergic processes in the striatum. Considering that the behavioral data from the cylinder test were not in line with the amphetamine-induced rotation data and no effects on dopaminergic cell bodies in the SNpc were observed, the neurorestorative potential of BT44 need to be confirmed in future experiments. It is recognized, that the aggressive lesion may have limited the neurorestorative effects of BT44 in our study.

Earlier studies have shown that striatal 6-OHDA lesion can downregulate GFRα1 and RET expression in the nigrostriatal pathway up to one month post lesion (Marco et al. 2002; Kozlowski et al. 2004; Gavin et al. 2014). Due to the fact that the delivery of the RET agonists occurred during this period of time, the downregulation of RET can negatively affect their neuroprotective efficacy. The extent of the receptor downregulation in the disease model would be an important factor to clarify in the future before proceeding into large-scale efficacy studies in order to gain better understanding of the potency of the experimental compounds *in vivo*. The ability of GDNF to halt the degeneration of dopaminergic cell bodies is facilitated by its retrograde transport to the SNpc via the remaining axons while there are no data demonstrating the retrograde transport of BT-

compounds (Lapchak et al. 1997; Tomac et al. 1995). Obviously, the considerably higher potency of GDNF as compared to BT-compounds as illustrated in Table 5.1 also accounts for its better efficacy in the neuroprotection and restoration experiments.

The unilateral 6-OHDA lesion model is based upon the imbalance of the nigrostriatal system between the hemispheres. The effects of unilaterally delivered interventions are compared to the intact side of the brain. Thus, this model is not optimal for testing the effects of lipid soluble compounds that readily spread to the contralateral hemisphere after unilateral administration and potentially elicit effects there too. BT-compounds tested in our studies are very lipophilic. Their putative diffusion to the control side during the seven- or 14-day infusion may compromise the read-outs that are based on measuring the asymmetry between the hemispheres.

6.6.2 Relevance of the behavioral tests in 6-OHDA lesion models

6.6.2.1 Correlation between amphetamine-induced turning rate and nigrostriatal integrity

Although amphetamine-induced rotation test has become the standard tool to monitor motor impairment and functional recovery in experiments with neuroprotective interventions, there are some important pitfalls that should be carefully considered in the future use of this test like Björklund and Dunnett (2019) have described in their critical reappraisal. We also analyzed the correlation between amphetamine-induced turning rate and the striatal TH-ir fiber density and nigral TH-ir cell number in the neurorestoration experiment (Study IV). In agreement with earlier reports, we did not find good correlations between these measures (Winkler et al. 1996; Kirik et al. 1998; Kozlowski et al. 2000; Voutilainen et al. 2009; Tronci et al. 2012; Björklund and Dunnett 2019).

Other neuronal circuits than nigrostriatal dopamine pathway may be responsible for the functional recovery seen in the experiment. For example, amphetamine induces dopamine release from dendritic vesicles in the SNr where dopamine primarily acts at presynaptic D1 receptors of the striatonigral MSNs facilitating GABA release (Rommelfanger and Wichmann 2010; Timmerman and Abercrombie 1996). This reduces the overactive firing of GABAergic SNr neurons projecting to the thalamus that is implicated in the motor imbalance of the unilateral 6-OHDA model. In our neurorestoration experiment, amphetamine-induced dendritic dopamine release in the SNr may have been augmented in GDNF-treated rats due to the significant preservation of dopaminergic cell bodies in the SNpc. Indeed, *in vivo* microdialysis measurements have also demonstrated enhanced amphetamine-evoked dopamine release in the SN of GDNF-treated rats (Hoffman et al. 1997). Thus, GDNF-induced significant functional improvement in animals with advanced dopaminergic denervation can be mediated by increased dopamine release in the striatal output structures. The sparse remaining striatal innervation may have a minor contribution in this case giving an explanation for the mismatch between the turning behavior and striatal fiber preservation.

In order to avoid amphetamine-evoked dopamine release in the extra-striatal structures as a possible confounding factor, it could have been beneficial to include apomorphine-induced rotational assessment in our behavioral test battery. The denervation of the striatum was at the level that is generally considered to be sufficient to cause supersensitization of the postsynaptic dopamine receptors which is a prerequisite for apomorphine-induced rotation test (Ungerstedt 1971). This test has been reported to correlate better with the loss of both striatal TH-ir fibers and nigral TH-ir cell bodies in 6-OHDA lesioned mice as compared to amphetamine-induced rotation test (Grealish et al. 2010). Apomorphine-induced turning behavior also had a better capacity to discriminate mice according to the extent of their nigrostriatal damage.

6.6.2.2 Other behavioral tests

Amphetamine-induced rotation test is a practical experiment to assess motor asymmetry and functional recovery in neuroprotection studies. PD patients, however, show a more complex repertoire of motor deficits than can be recapitulated with simple drug-induced motor tests. Therefore, behavioral tests allowing quantification of spontaneous motor and sensorimotor functions would provide more informative outcomes (Dunnett 2005). It is recommended to combine amphetamine-induced rotation test with at least one other test measuring spontaneous motor behavior (Björklund and Dunnett 2019). For 6-OHDA lesioned rats, paw-use asymmetry measured with the cylinder, stepping or staircase test, and for 6-OHDA lesioned mice e.g. sensorimotor behavior in the corridor test, would be profitable adjuncts.

For these reasons, we also analyzed spontaneous forepaw-use asymmetry using the cylinder test in our neurorestoration experiment (Study IV). Despite significant recovery in the amphetamine-induced turning behavior, neither GDNF nor BT44 were able to normalize forepaw use in the cylinder test. These data are in accordance with earlier reports where GDNF protein injection or gene therapy into 6-OHDA-lesioned striatum significantly alleviated amphetamine-induced turning behavior but showed no effects in the cylinder test (Georgievska et al. 2002; Gasmi et al. 2007b; Yue et al. 2014). Also in 6-OHDA lesioned mice, the behavioral deficits observed in the cylinder test showed poor correlation with amphetamine-induced turning behavior as well as with the integrity of the nigrostriatal pathway (Grealish et al. 2010). It should be noted, that not only the total extent of striatal dopaminergic denervation, but also its regional distribution affects the functional output and magnitude of the motor impairment (Kirik et al. 1998; Björklund and Dunnett 2019). Amphetamine-induced turning rate is known to be particularly sensitive to changes in dopaminergic innervation within the dorsomedial and dorsolateral parts of the striatum, whereas the ventrolateral parts of the striatum have more pronounced control on movement initiation, sensorimotor orientation and skilled motor behavior. Since our treatment infusions were directed into the dorsomedial striatum, they might have had limited effects on the ventrolateral striatum, and thus on spontaneous forepaw use in the cylinder test.

Notably, most of the behavioral tests used to assess the non-motor symptoms of PD are not particularly designed for parkinsonian animals (Asakawa et al. 2016). We are lacking specific tools for the assessment of non-motor symptoms especially in rodent models of PD. Development of such behavioral tests would be essential for the advancement of this important, but rather

neglected field of research. Apart from the behavioral test, also other approaches can be considered to follow longitudinal functional changes in animal models of PD. For example, *in vivo* electrophysiology offers another approach to detect functional changes in various neuronal circuits in freely-moving animals.

6.6.3 Validity of the disease models

The encouraging preclinical results with NTF-based therapies have failed to translate into positive outcomes in clinical trials. To enable successful clinical translation of disease-modifying treatments, we should take a critical look at the limitations of the current preclinical models. Are the results obtained with these models relevant in terms of the clinical condition? As experimental animals do not naturally develop PD, the disease has to be artificially induced by targeting one or few pathological processes of the disease.

A major obstacle in developing effective disease-modifying therapies for PD is the lack of animal models that fully recapitulate the pathological and functional disease progression and the phenotype of PD (Paul and Sullivan 2018). The current animal models typically mimic only one or few aspects of the disease. The traditional neurotoxin-based (e.g. 6-OHDA and MPTP) animal models, for example, reproduce the degeneration of nigrostriatal dopamine neurons and neuroinflammation but fail to replicate several other crucial features such as the accumulation of α-syn containing protein inclusions. The mechanism of the neuronal insult is also different from the human disease. One of the important characteristics of human PD is that the pathology progresses over decades. Neurotoxins, instead, produce an acute lesion leaving little time for compensatory mechanisms to take place. The genetic animal models of PD seem to exhibit good construct validity as they are based on mutations that cause genetic forms of the human disease. However, they fail to produce a clear parkinsonian phenotype in terms of neurodegeneration and motor symptoms.

Traditionally, the neurotoxin-induced animal models are focused on insulting the nigrostriatal dopamine neurons. This, indeed, offers a good approach to model motor dysfunctions but does not take into consideration the widespread nature of the disease. PD pathology is not restricted to the nigrostriatal dopamine system but affects multiple neuronal populations and neurotransmitter systems, which also give rise to the numerous non-motor symptoms of PD and should be better considered in preclinical drug development (Olanow et al. 2015b). Overall, the neurotoxin models serve as good tools for testing symptomatic treatments but might not be as useful in the development of disease-modifying therapies.

One common issue with the rodent models of PD is the routine use of young adult animals as experimental subjects. Metabolism, plasticity and neuronal function in the young brain differ from those of the aged brain which makes the translation to PD patients even harder. Perhaps, establishment of disease models in aged animals could partly help to overcome this translatability problem. Another common problem relates to the timing of a neurotrophic therapy (Paul and Sullivan 2018). Studies where the therapy is administered after the nigrostriatal pathway has already degenerated, like in our neurorestoration experiment (Study IV), are more relevant to the

clinical scenario where a substantial nigrostriatal damage already exists at the time when the therapy can be initiated. In the majority of the preclinical studies, however, NTFs have been administered before or shortly after the lesion. Thus, there is a mismatch with clinical trials where the NTF interventions are mostly tested in patients with advanced PD.

Since most animal models tend to reproduce only few features of human PD, it is essential to test potential treatments in a combination of different disease models. In addition to neurotoxin-based models, α-syn-based models should be part of the preclinical testing. Neuroinflammation, as one important aspect of the PD pathobiology, also deserves more attention. Furthermore, dopamine neurons generated from PD patient-derived induced pluripotent stem (iPS) cells should be included in the battery of preclinical PD models. They harbor the full human-specific metabolic and regulatory pathways, and thus provide a valuable tool to investigate dopamine metabolism and mechanisms of dopaminergic degeneration (Meiser et al. 2013). Other possible platforms to be used in drug development include, for example, organoids, 3D cell constructs and organ-on-a-chips. The importance of such new platforms is growing, and they will help us to replace some of the *in vivo* experiments according to 3R principles.

If neurotrophic therapies will enter the clinical use, they will most probably be used in combination with standard PD medications. Therefore, it is of outmost importance to evaluate their efficacy in animal models together with other anti-parkinsonian treatments. For example, GDNF delivery into the lateral ventricle of MPTP-lesioned marmosets was shown to reduce the severity of L-DOPA-induced dyskinesias (Iravani et al. 2001). These type of co-administration experiments are warranted to improve the translatability of preclinical NTF studies.

6.7 General discussion

PD is a common neurodegenerative disorder with complex pathobiology and multitude of genetic and environmental risk factors (Kalia and Lang 2015). The diagnosis is preceded by a several years or even decades long prodromal phase, during which the characteristic parkinsonian Lewy pathology progresses from the periphery into the brain. Eventually, patients become afflicted by cardinal motor symptoms that arise from the progressive loss of nigrostriatal dopamine neurons. Equally importantly, several other neuronal populations are also affected which contributes to various non-motor symptoms of PD. The current treatments mainly target the motor impairment by replenishing reduced dopaminergic neurotransmission within the striatum. None of them, however, is capable to modify the neurodegenerative process or slow down the progression of the disease. Novel disease-modifying agents are urgently needed. NTFs and their biological effects mimicking small molecule compounds hold promise for this quest, but more preclinical work is required to further characterize their mechanisms of action, confirm their neuroprotective potential and uncover their effects on other brain cells than nigrostriatal dopamine neurons. It is not clear, for example, whether exogenous NTFs directed to the nigrostriatal dopamine system have significant effects on the non-motor symptoms of PD. Systemically administered NTF mimetics with widespread target engagement in all afflicted brain areas could potentially help to tackle also these disabling features of the disease.

PD is a clinically heterogeneous disorder. There are many ways to classify various subtypes of PD, for example based on motor symptoms (e.g. tremor-dominant vs. non-tremor-dominant), cognitive features, age at onset, rate of progression or various combinations of these (Marras and Lang 2013). The different subtypes are believed to have distinct etiologies and pathogenesis. Better understanding of those would allow the identification of novel therapeutic targets and open avenues for the development of effective and safe disease-modifying therapies.

GDNF and NRTN have been considered as the most promising candidates for the first neurotrophic therapy for PD based on the solid body of preclinical evidence. Failures in the phase II clinical trials, however, have raised questions whether RET is an optimal target receptor. In PD models induced by viral vector-mediated overexpression of α-syn, gene delivery of GDNF to the SNpc had no effect on the survival of dopamine neurons or motor impairment (Lo Bianco et al. 2004; Decressac et al. 2011). Importantly, α-syn overexpression was shown to downregulate the transcription factor Nurr1 and its downstream target RET diminishing the intracellular response to GDNF (Decressac et al. 2012). Nurr1 and RET expression was also found to be reduced in nigral neurons in PD patients suggesting that α-syn-induced downregulation of RET might account for the negative results of GDNF in the α-syn overexpressing animal models and its limited efficacy in the clinical trials (Chu et al. 2006; Decressac et al. 2012). It should be noted, however, that there are also contrasting reports showing no downregulation of Nurr1 or RET in PD patients (Bäckman et al. 2006; Su et al. 2017; Walker et al. 1998). Formation of α-syn aggregates may also impede the axonal transport of GDNF like it has been shown to impair the transportation of BDNF signaling endosomes (Chung et al. 2009; Volpicelli-Daley et al. 2014). This is an important factor to consider when planning the future experiments and deciding the administration sites of NTFs.

Gain-of-function mutations in *Ret* gene causing the inherited cancer syndromes MEN2 and familial medullary thyroid carcinoma have triggered concerns regarding the oncogenic potential of RET activation (Plaza-Menacho et al. 2006). However, with an intermittent dosing scheme, the activation of RET would not be continuous like in the RET-related cancers which would help to manage the risk for tumors. Important to note, that in a recent study investigating the long-term effects of GDNF overexpression from the endogenous locus, continuously increased activation of GDNF-RET signaling did not elicit adverse effects or tumors (Turconi et al. 2020). Intermittent delivery also results in smaller cumulative doses as compared to continuous dosing schemes (Whone et al. 2019). This further helps to avoid potential safety risks. Target engagement in a pulsatile fashion may also offer benefits over continuous receptor stimulation in terms of ligand-dependent receptor desensitization that potentially reduces the responsiveness of target tissues to a NTF therapy (Lohse 1993).

Due to the abovementioned issues, it is vital to continue looking also for other, RET-independent opportunities for a disease-modifying therapy. Here, unconventional NTFs CDNF and MANF have shown great promise in preclinical studies. However, our knowledge of their biological role and mechanism(s) of action is still limited (Lindahl et al. 2017). The discovery of their putative receptors would enable better understanding of their physiological functions and development of new neurotrophic candidates based on their signaling mechanisms.

6.7.1 Importance of biomarker development

It is imperative to develop better diagnostic tools and novel biomarkers in parallel with disease-modifying therapies to allow earlier intervention with neuroprotective agents, and consequently an improved therapeutic outcome. With the current diagnostic criteria, PD is identified only upon the manifestation of the motor symptoms which occurs several years after the neurodegenerative process has started (Miller and O'Callaghan 2015; Parnetti et al. 2019). This hinders the attempts to intervene at the earliest phase of the disease when there is still a sufficient number of surviving dopaminergic neurons and axons that are capable to respond to the therapy. A reliable prodromal biomarker could provide a prolonged time frame, during which disease-modifying candidates could be administered to halt or slow down the cell loss. Currently, this time frame is not very long from the diagnosis to virtually complete loss of dopaminergic fibers in the putamen (4 to 5 years) (Burke and O'Malley 2013; Kordower et al. 2013). The earlier the NTF-based therapy can be initiated, the better efficacy it is expected to have as was demonstrated in the clinical trial with AAV2-NRTN (Olanow et al. 2015). In an effort to enable enrolment of patients at the earliest stage of PD into clinical trials of neuroprotective therapies, the International Parkinson and Movement Disorder Society task force has developed research criteria for prodromal PD (Berg et al. 2015). Identification of individuals in the prodromal phase would also facilitate the clarification of the disease mechanisms and progression. On the other hand, there is a greater risk for misdiagnosis, particularly with atypical parkinsonian syndromes, at the early stages of the disease (Tolosa et al. 2006).

PD should not be conceptualized as a unitary disorder (Berg et al. 2014). It comprises a spectrum of subtypes with distinct etiologies, pathological processes and therapeutic needs. Validated biomarkers would help to stratify patients according to their individual form of the disease. More precise diagnosis would pave the way for better understanding of the pathological processes in each subtype and more personalized disease management. Importantly, disease subtyping would contribute to the development of more translational disease models and design of more successful clinical trials with stratified inclusion criteria. The failed attempts to find neuroprotective strategies for PD may stem from the reductionist approach in the conducted clinical trials which have paid little attention to the variability of the disease at the individual level (Espay et al. 2017).

Currently, UPDRS is used for longitudinal follow-up of the disease progression based on episodic and subjective clinical evaluations. Thus, drug development decisions in clinical trials rely on sparse datapoints contributing to frequent and costly failures. There is a need for reliable longitudinal biomarkers. Development of digital biomarkers, for example, may help to address many of the current diagnostic shortcomings in an economical fashion. They would allow an objective approach to continuously track fluctuations in motor and non-motor symptoms during patients' daily life. The resulting rich real-world datasets may prove to be highly predictive in assessing clinical improvement in PD studies and permit personalized therapeutic adaptations.

Ideally, preclinical drug development would already be accompanied with a translatable biomarker that could be followed through the whole process from animal models to clinical trials

and regular patient monitoring. This kind of translatable biomarker would help to predict the effects of an intervention in a patient population based on preclinical tests in animal models. For example, imaging modalities would be suitable for translatable biomarkers because they provide a direct approach to measure specific neurofunctional properties and the same methodology can be applied both to experimental animals and humans.

6.7.2 Issues with the clinical studies

Despite promising outcomes in preclinical studies and open-label phase I trials, none of the controlled double-blind phase II trials have reported clear clinical benefit for intracranial GDNF or NRTN therapy in patients with moderate or advanced PD (Hegarty et al. 2017). Negative outcomes from these proof-of-concept studies have raised questions whether NTFs in general are translatable into an effective disease-modifying therapy or whether the trials have been conducted sub-optimally. If the NTF hypothesis is still valid, the limitations of the studies should be carefully appraised.

The importance of conducting placebo-controlled double-blind trials should be underlined. Marked placebo effect is a well-described phenomenon in PD trials, especially when neurosurgical procedures are involved, which may explain the efficacy differences between the open-label and controlled double-blind trials (Goetz et al. 2008; Alterman et al. 2011). Therefore, no conclusions should be drawn from the open-label studies that are not properly controlled for the placebo effect.

Patients enrolled in the phase II trials may have been too advanced to benefit from the NTF therapy (Olanow et al. 2015). Dopamine neurons are so profoundly degenerated and dysfunctional at the later stages of PD that they might not be capable of responding to the therapy anymore. Indeed, greater benefits have been observed in a subgroup of patients who received AAV2-NRTN therapy less than five years after the diagnosis (Bartus 2015; Olanow et al. 2015). In the future, it might be necessary to recruit patients at an earlier stage of the disease which complicates weighing the risks against the benefits.

Transport of intraputamenally delivered GDNF or NRTN to the dopaminergic cell bodies in the SNpc is thought be necessary for their pro-survival effects. Thus, the feasibility of treating advanced PD patients with these factors is questioned because the putamenal innervation is almost completely disappeared within 4-5 years post diagnosis (Kordower et al. 2013). This denervation potentially contributes to the impaired retrograde axonal transport, as reported in AAV2-NRTN-treated patients enrolled in the clinical trial more than five years post diagnosis (Bartus et al. 2011; Bartus et al. 2015).

There are some indications of clinical benefits at the later time points of the trials suggesting delayed and sustained effects for NTFs (Love et al. 2005; Marks et al. 2010; Patel et al. 2005). Thus, a longer-term follow-up might be required to detect possible clinical improvements. Ethical aspects, however, need to be considered in order to avoid excessively long exposure to placebo in a surgical setting.

It has been suggested that technical differences in the drug delivery systems and infusion protocols partly contributed to the varying results between the phase I and phase II trials (Salvatore et al. 2006; Sherer et al. 2006; Morrison et al. 2007). Furthermore, explanations for the lack of efficacy in the phase II trials can also include insufficient dosage, heterogeneous or inadequate distribution of GDNF and NRTN in the target tissue and formation of neutralizing antibodies (Tatarewicz et al. 2007).

6.7.3 Future directions

Despite holding great therapeutic potential for PD, it might well be that NTFs are not sufficient alone to combat neurodegeneration and restore neuronal function. Combination therapies may offer an opportunity to maximize the benefits of NTFs. Coadministration of different NTFs seems to be more efficient in protecting, repairing and activating dopamine neurons than the delivery of a single NTF (Voutilainen et al. 2017). NTFs could provide additive therapeutic benefit also when administered in conjunction with other treatment strategies. Usually, synergistic therapies help to reduce the dose of each individual treatment, and thus mitigate the risk for possible adverse effects.

It is becoming evident that the underlying pathological processes such as Lewy pathology or the augmented neuroinflammatory state have to be resolved in order to achieve favorable conditions for neuroregeneration. Only then, neurotrophic therapies would have a better opportunity to promote neuronal survival. Thus, it would be worthwhile to study the effects of NTFs in conjunction with, for example, experimental anti-α-syn antibodies that reduce the spread of α-syn aggregates in the brain. Chronic neuroinflammation is one of the key elements in PD pathogenesis contributing to the neuronal dysfunction and degeneration. Reactive microglia are the primary mediators of neuroinflammation. Astrocytes, in turn, regulate the microglial responses by secreting factors that reduce their phagocytic activity and production of ROS. Therefore, combining anti-inflammatory (or immunomodulatory) treatments with trophic factors may open new avenues to induce regenerative processes in PD. Interestingly, experiments in cultured midbrain microglial cells identified GDNF as one of the major astrocyte-derived factors regulating their activation in a GFRα1 receptor -dependent manner (Rocha et al. 2012). CDNF and MANF have also been shown to reduce the secretion of pro-inflammatory cytokines from glial cells suggesting an important regulatory role in neuroinflammation (Zhao et al. 2013; Zhu et al. 2016; Neves et al. 2016). Thus, they might provide synergistic benefits if administered together with other NTFs (Voutilainen et al. 2017).

The promising safety outcomes in the first-in-human study with CDNF encourage its further clinical development. It will be most interesting to see quantitative data on the clinical efficacy of intraputamenally delivered CDNF in PD patients. Our Studies I and II provide support for the clinical development of CDNF and MANF. The main outcomes in Study I suggest that NTFs have diverging effects on dopamine release and metabolizing enzymes in the normal rat brain. It was promising to see that CDNF and MANF did not decrease dopaminergic neurotransmission in the striatum. Thus, they would be most probably compatible with the current dopaminergic medications of PD. Another important finding was the COMT activity increasing effect of GDNF which calls for

attention in possible clinical trials in the future in terms of the compatibility of GDNF therapy with the existing medications of patients, in particularly with COMT-inhibitors. More extensive preclinical experiments determining the effects of NTFs on dopamine neurochemistry in a time-dependent manner should be performed. In addition, their coadministration together with standard symptomatic PD medications should be studied in animal models of PD to support the clinical development.

NTFs are protein therapeutics which complicates their clinical use and restricts them to more advanced disease conditions due to the need for intracranial administration. The initiation of the treatment at a later stage of the disease can potentially reduce their neuroprotective potential. To overcome these limitations, we have developed small molecule NTF mimetics that could be suitable for peripheral administration at an earlier phase of the disease. Indeed, BT13 and BT44 seemed to induce functional recovery in one commonly used animal model of PD when infused into the brain at the time or few weeks after the neurotoxin lesion (Studies III and IV). These RET signaling activating compounds serve as promising leads that deserve further development. First, their pharmacochemical properties should be improved to enable better solubility into aqueous vehicles and longer half-life in the brain. Secondly, their potency should be increased in order to avoid need for micromolar concentrations, and consequently reduce the risk for unspecific off-target effects. Higher efficacy would also be desirable in order to better reveal their neuroprotective potential. Lastly, the mechanism of direct RET agonism reduces their specificity and increases the risk for antagonized endogenous GFL-GFRα signaling. Therefore, the development of the next generation compounds towards GFRα PAMs would most likely improve their specificity and safety profile.

With the improved GFL-mimicking drug candidates, the neuroprotective efficacy after peripheral administration should be tested in proper dose-response studies using several well-validated PD models to gain a more comprehensive picture of their effects on PD pathology. A critical question that remains is whether they would work in α-syn-based PD models where Nurr1 and RET may be downregulated (Decressac et al. 2012). As we learned from Studies III and IV, the behavioral read-outs did not correlate optimally with the histological read-outs measuring the integrity of the nigrostriatal dopamine system. Therefore, more attention should be paid to validate the animal models and behavioral assessments properly in pilot experiments before applying them into large-scale *in vivo* studies.

7 CONCLUSIONS

The main objective of this work was to clarify the functional effects of intracranially delivered GDNF, CDNF and MANF on the nigrostriatal dopamine system in the normal rat brain and to examine the neuroprotective potential of novel small molecule RET agonists in a 6-OHDA rat model of PD. Generally, the results support the preclinical characterization of CDNF, MANF and RET agonists as potential neurotrophic therapies for PD.

The principal findings and conclusions of this thesis are:

1. Intrastriatal injection of MANF enhances stimulus-evoked dopamine release and DOPAC/dopamine turnover in the striatum of normal freely-moving rats. GDNF-injection, instead, increases *in vivo* TH and COMT activity and decreases MAO-A activity in the striatum of normal rats. These divergent and long-lasting effects of exogenously administered NTFs on dopamine neurochemistry are noteworthy when considering their compatibility of with the existing dopaminergic medications of the patients.

2. CDNF readily diffuses in the brainstem but is not transported to distal brain areas after an injection to the SN of intact rats. Nigrally injected CDNF also colocalizes with TH-ir, but not with PV-ir, neurons in the SN. These data suggest dopaminergic selectivity of CDNF and facilitate the selection of optimal delivery site and protocol for CDNF.

3. Small molecule RET agonists BT13 and BT44 induce the phosphorylation of RET and activate the downstream pro-survival signaling pathways Akt and ERK. They promote the survival of cultured midbrain dopamine neurons in a RET-dependent manner. BT13 stimulates striatal dopamine release *in vivo* and improves functional recovery in a 6-OHDA rat model of PD. BT44 alleviates amphetamine-induced motor dysfunction in 6-OHDA lesioned rats and shows potential to restore striatal TH-ir fibers. Unlike GFLs, BT13 and BT44 are able to cross the BBB. They serve as promising lead compounds but require further drug design and development in terms of their efficacy and pharmacochemical properties.

Taken together, these results shed light on the functional effects and distribution properties of exogenously administered NTFs on the nigrostriatal dopamine system in the intact rat brain. This work also provides a proof-of-principle for small molecule RET agonists mimicking the neurotrophic effects of GFLs in an animal model of PD.

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